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MARLOS BARBOSA RIBEIRO

ESTUDO CLÍNICO DO CONTEÚDO INFLAMATÓRIO E INFECCIOSO DE DENTES COM INSUCESSO DO TRATAMENTO ENDODÔNTICO

CLINICAL STUDY OF THE INFLAMMATORY AND INFECTIOUS CONTENT OF TEETH WITH FAILURE OF ENDODONTIC TREATMENT

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Tese apresentada à Faculdade de Odontologia de Piracicaba da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do título de Doutor em Clínica Odontológica, na Área de Endodontia.

Thesis presented to the Piracicaba Dental School of the University of Campinas in partial fulfillment of the requirements for the degree of Doctor, in the area of Endodontics.

Orientadora: Profa. Dra. Brenda Paula Figueiredo de Almeida Gomes

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A Ata da defesa com as respectivas assinaturas dos membros encontra-se no processo de vida acadêmica do aluno.

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"A sabedoria é um paradoxo. O homem que mais sabe é aquele que mais reconhece a vastidão da sua ignorância"

Friedrich Wilhelm Nietzche

RESUMO

Introdução: O conteúdo infeccioso dos canais radiculares, incluindo bactérias e seus bio-produtos como o LTA (ácido lipoteicóico), e produtos derivados de genes de virulência, podem induzir a liberação de citocinas pró-inflamatórias (CPI) e metaloproteinases de matriz (MMPs) causando injuria aos tecidos periapicais. Os objetivos deste estudo foram: **(Capítulo I)** a) caracterizar os microrganismos Gram-positivos e estabelecer a prevalência de *Enterococcus faecalis* nas diferentes fases da terapia endodôntica; b) investigar a presença de LTA; **(Capítulo II)** c) monitorar *in vivo* o efeito do preparo químico-mecânico (PQM) e da medicação intracanal (MIC) na redução de bactérias cultiváveis (BC), LTA, CPI (TNF-α e IL1-β) e MMPs (-2, -3, -8, -9 e -13) em canais radiculares de dentes com periodontite apical pós-tratamento endodôntico; **(Capítulo III)** d) investigar a suscetibilidade antimicrobiana de *E. faecalis* isolados e a prevalência dos seus fatores de virulência.

Metodologia: Vinte canais radiculares infectados de dentes unirradiculares foram divididos aleatoriamente em dois grupos de acordo com a substância química auxiliar (SQA) utilizada durante o PQM (n = 10 por grupo): G1 - clorexidina (CLX) 2% gel e G2 - hipoclorito de sódio (NaOCl) 6%. As amostras do conteúdo infeccioso e inflamatório dos canais radiculares foram obtidas usando cones de papel estéreis antes (C1) e após (C2) o PQM e após 30 dias com MIC ($Ca[OH]_2$ + clorexidina 2% gel) (C3). **(Capítulo I)** A identificação microbiana foi realizada por testes bioquímicos; BC foram determinadas por contagem das unidades formadoras de colônia (UFC/mL); os níveis de LTA foram mensurados usando o ensaio imunoenzimático (ELISA) (pg/mL); **(Capítulo II)** CPIs e MMPs foram também mensuradas usando o ensaio imunoenzimático (ELISA) (pg/mL); **(Capítulo III)** a suscetibilidade antimicrobiana de diferentes antibióticos foi determinada pela concentração inibidora mínima (CIM) utilizando o método do E-test. Os genes de virulência (*ace, asa, asa373, cylA, efaA, esp* e *gelE*) dos *E. faecalis* isolados foram detectados através da técnica de PCR.

Resultados: **(Capítulo I)** 82 espécies bacterianas Gram-positivas, de um total de 102 bactérias isoladas, foram encontradas nos canais radiculares (62 na C1, 4 na C2 e 16 na C3). Os gêneros mais prevalentes foram *Actinomyces* spp, *Aerococcus* spp, *Enterococcus* spp, *Gemella* spp and *Staphylococcus* spp. *E. faecalis* foi a bacteria mais prevalente. BC (101,2±79,2), LTA (94,1±61,7), **(Capítulo II)** CPI (TNF-

α: 8,8±4,7 e IL1-β: 1,2±0,4) e MMPs (MMP-2: 803,7±96,4; MMP-3: 453,9±229,3; MMP-8: 245,9±122,4; MMP-9: 129,4±29,6 e MMP-13: 70,8±12,8) estavam presentes em todas as amostras (C1). Foi encontrada diminuição dos níveis globais de BC, LTA, CPI e MMPs, em todos os grupos (P < 0,05) na C2. **(Capítulos I e II)** O percentual de redução na C2 foi o seguinte: BC (94,4%), LTA (60,8%), CPI (TNF-α: 89,7% e IL1-β: 91,6%) e MMPs (MMP-2: 7,8%; MMP-3: 30,3%; MMP-8: 6,9%; MMP-9: 6% e MMP-13: 13,9%) (P < 0,05). Não houve diferença estatística entre as SQA testadas na redução do conteúdo infeccioso e inflamatório na C2 (P > 0,05). O percentual de redução na C3 foi o seguinte: BC (16,7%) (P > 0,05), LTA (39%) (P < 0,05) e MMP-8 (2,4%) (P > 0,05). Por outro lado, observou-se aumento de TNF-α, IL1-β, MMP-2 (P < 0,05), MMP-3, MMP-9 e MMP-13 (P > 0,05) na C3. A MIC foi efetiva na redução de LTA no G1; e BC, MMP-3 e MMP-8 no G2 (P < 0,05). No entanto, houve aumento de todas as CPIs e MMPs (-3, -8, -9 e -13) no G1 e (-2 e -13) no G2 (P < 0,05). **(Capítulo III)** Amoxicilina + ácido clavulânico foi efetivo contra todas as cepas. Resistência intermediária de algumas cepas foi observada para Amoxicilina (5%), Azitromicina (20%), Benzilpenicilina (5%), Ciprofloxacina (15%), Doxiciclina (5%), Eritromicina (75%), Tetraciclina (10%) e Vancomicina (15%). Total resistência a Clindamicina (60%), Clorafenicol (5%), Gentamicina (65%), Metronidazol (95%), Moxifloxacina (5%) e Rifampicina (10%) foi observada para algumas cepas. Em relação aos fatores de virulência dos *E. faecalis* isolados, houve detecção para *ace* (100%), *asa* (60%), *asa373* (15%), *esp* (70%) e *gelE* (75%). Em contrapartida, os genes *cylA* e *efaA* não foram detectados.

Conclusão: **(Capítulos I e II)** Independente da utilização de CLX ou NaOCl, o PQM é efetivo na redução do conteúdo infeccioso/inflamatório de canais radiculares de dentes com insucesso do tratamento endodôntico. A MIC elevou os níveis de TNF-α, IL-1β e MMP-2. **(Capítulo III)** Amoxicilina + Ác. Clavulânico mostrou atividade antimicrobiana para todas as cepas de *E faecalis* testadas, enquanto que alguns isolados apresentaram resistência a Clindamicina, Clorafenicol, Gentamicina, Metronidazol, Moxifloxacina e Rifampicina. Todas as cepas de *E. faecalis* apresentaram os genes *ace, asa, asa373, esp* e *gelE*.

Palavras-chave: Bactérias. Clorexidina. Hipoclorito de sódio. Enterococcus faecalis. Testes de sensibilidade microbiana. Fatores de virulência. Citocinas. Metaloproteinases da matriz.

ABSTRACT

Introduction: The infectious contents of root canals, including bacteria and their by-products such as LTA (lipoteichoic acid) and derived products of virulence genes can induce the release of proinflammatory cytokines (PIC) and matrix metalloproteinases (MMP), causing injuries to the periapical tissues. The aims of this study were: (**Chapter I**) a) to characterize the Gram-positive microorganisms and to establish the prevalence of *Enterococcus faecalis* in the different phases of the endodontic therapy; b) to investigate the presence of LTA, (**Chapter II**) PIC and MMP, and also to monitor *in vivo* the effect of chemomechanical preparation (CMP) and intracanal medication (ICM) on the reduction of cultivable bacteria (CB), LTA, PIC (TNF-α and IL1-β) and MMPs (-2, -3, -8, -9 and -13) in the root canals of teeth with post-treatment apical periodontitis; and (**Chapter III**) c) to investigate the prevalence of virulence factors and the antimicrobial susceptibility of *E. faecalis* isolates.

Methods: Twenty infected root canals of single-rooted teeth were randomly assigned into two groups according to the irrigant used during CMP (n = 10 *per* group): G1 - 2% chlorhexidine (CHX) gel and G2 - 6% sodium hypochlorite (NaOCl). Root canal contents were taken by using paper points before (S1) and after CMP (S2) and after 30 days of ICM ($Ca[OH]_2$ + 2% CHX gel) (S3). (**Chapter I**) The microbial identification was performed using biochemical tests; CB was determined by counting the colony-forming unit (CFU/mL); LTA levels were measured using enzyme-linked immunosorbent assay (pg/mL);, (**Chapter II**) PICs and MMPs levels were measured by using enzyme-linked immunosorbent assay (pg/mL); (**Chapter III**) the antimicrobial susceptibility of different antibiotics was determined by the minimum inhibitory concentration (MIC) by using the E-test method. *E. faecalis* virulence factors (*ace, asa, asa373, cylA, efaA, esp* and *gelE*) were detected by PCR assay.

Results: (**Chapter I**) A total of 82 Gram-positive bacteria species, out of 102 bacteria isolated, were found in the root canals (62 in S1, 4 in S2 and 16 in S3). The most prevalent genera were *Actinomyces* spp, *Aerococcus* spp, *Enterococcus* spp, *Gemella* spp and *Staphylococcus* spp. *E. faecalis* was the most frequent bacteria isolated. CB (101.2±79.2), LTA (94.1±61.7), (**Chapter II**) PICs (TNF-α: 8.8±4.7 and IL1-β: 1.2±0.4) and MMPs (MMP-2: 803.7±96.4; MMP-3: 453.9±229.3; MMP-8: 245.9±122.4; MMP-9: 129.4±29.6 and MMP-13: 70.8±12.8) were present in all S1 samples. Decrease of the overall levels was found in all groups ($P < 0.05$) in S2

samples. (**Chapters I and II**) Reduction percentage in S2 was the following: CB (94.4%), LTA (60.8%), PIC (TNF-α: 89.7% and lL1-β: 91.6%) and MMPs (MMP-2: 7.8%; MMP-3: 30.3%; MMP-8: 6.9%; MMP-9: 6% and MMP-13: 13.9%) ($P < 0.05$). There was no difference between the chemical substances tested on the infectious content reduction in S2 ($P > 0.05$). Reduction percentage in S3 was the following: CB (16.7%) ($P > 0.05$), LTA (39%) ($P < .05$), and MMP-8: (2.4%) ($P > 0.05$). On the other hand, there was an increase of TNF-α, lL1-β, MMP-2 ($P < 0.05$), MMP-3, MMP-9 and MMP-13 ($P > 0.05$) levels in S3. lCM was effective in reducing LTA in G1; and CB, MMP-3 and MM-8 in G2 ($P < 0.05$). However, there was an increase of all PCI and MMPs in S3 for G1 (-3, -8, -9 and -13) and G2 (-2 and -13) ($P < 0.05$). (**Chapter III**) Amoxicilin + clavulanate was effective against the all strains. Intermediate resistance of some strains was observed for Amoxicillin (5%), Azithromycin (20%), Benzylpenicillin (5%), Ciprofloxacin (15%), Doxycycline (5%), Erythromycin (75%), Tetracycline (10%) and Vancomycin (15%). A total resistance to Clindamycin (60%), Chloramphenicol (5%), Gentamicin (65%), Metronidazole (95%), Moxifloxacin (5%) and Rifampicin (10%) was observed for some strains. In relation to the virulence factors of *E. faecalis* isolates, there was detection of *ace* (100%), *asa* (60%), *asa373* (15%), *esp* (70%) and *gelE* (75%) genes. However, *cylA* and *efaA* genes were not detected.

Conclusion: (**Chapters I and II**) Regardless the use of NaOCl or CHX, CMP is effective in reducing the infectious/inflammatory content of the root canals of teeth with endodontic treatment failure. The MIC increased the levels of TNF-α, lL-1β and MMP-2. (**Chapter III**) Amoxicillin + Clavulanate showed antimicrobial activity against all strains of E faecalis tested, while some isolates showed resistance to Clindamycin, Chloramphenicol, Gentamicin, Metronidazole, Moxifloxacin and Rifampicin. All strains of *E. faecalis* had the genes *ace, asa, asa373, esp* and *gelE*.

Keywords: Bacteria. Chlorhexidine. Sodium hypochlorite. Enterococcus faecalis. Cytokines. Matrix metalloproteinases. Microbial sensitivity tests. Virulence factor.

SUMÁRIO

INTRODUÇÃO

As elevadas taxas de sucesso do tratamento endodôntico se devem ao avanço das técnicas e materiais utilizados, como também ao aumento do número de profissionais especializados no mercado. No entanto, diversos fatores intrínsecos e/ou extrísecos podem influenciar de maneira negativa no tratamento e conduzi-lo ao fracasso (Occhi et al., 2011; Margarit et al., 2012; Estrela et al., 2014; Wong et al, 2014). Por isso é importante destacar que mesmo que o percentual de insucessos seja baixo (Estrela et al., 2014; Wong et al., 2014), os tratamentos estão sujeitos a falhas.

O principal fator de insucesso endodôntico é a infecção intrarradicular (Nair et al., 1990; Siqueira Jr, 2001; Schirrmeister et al., 2009; Endo et al., 2012, 2013; Rahimi et al., 2014). Microrganismos podem se organizar na forma de biofilme e serem capazes de resistir aos procedimentos intracanais (Nair et al., 1999; Distel et al., 2002; Wang et al., 2012), levando à falha da terapia. As áreas não atingidas durante o preparo químico-mecânico (PQM) são favoráveis à manutenção deste biofilme, contribuindo para a persistência ou reinfecção intraradicular, seja devido ao dano tecidual direto causado pelos microrganismos, ou pela ativação de uma rede de mediadores químicos capazes de perpetuar um processo inflamatório crônico na região periapical, caracterizado como periodontite apical (Lin et al., 1991; Chugal et al., 2003; Peters et al., 2004; Occhi et al., 2011; Endo et al., 2013; Siqueira Jr et al., 2014).

Os microrganismos são os principais agentes etiológicos da periodontite apical (Kakehashi et al., 1965; Gomes et al., 1996; Rocas et al., 2010; Siqueira Jr et al., 2011; Rahimi et al, 2014) e possuem diferentes mecanismos de resistência e virulência capazes induzir ou perpetuar um processo inflamatório na região periapical após obturação de canal (Siqueira Jr & Rocas, 2007; Dos Santos et al, 2014.; Zhao et al., 2014). Dentre eles, destaca a resistência a antibióticos utilizados na prática endodôntica (Costa, Souza-Filho & Barbosa, 2003), formação de biofilme (Siqueira Jr & Rocas, 2007; Baik et al., 2008; Lee & Baek, 2012), expressão de genes de virulência (Baik et al., 2008; Lee & Baek, 2012), liberação de fatores de virulência (Ginsburg, 2002; Hermann et al., 2002; Hahn e Liewehr, 2007; Baik et al., 2008; Ryu et al., 2009), e características de patogenicidade (Kayaoglu e & Ørstavik, 2004).

A microbiota de um dente com insucesso endodôntico é diferente daquela relacionada a infecção primária, com predomínio de microrganismos anaeróbios facultativos, como os gênero *Actinomyces*, *Candida* e Enterococos (Pinheiro et al, 2003a,b; Endo et al., 2012, 2013). *Enterococcus faecalis* é bactéria mais freqüentemente detectada e é capaz de suportar grande variação de pH, temperatura e tensão de O_2 no interior do canal radicular (Baik et al., 2008; Gomes et al., 2008; Lee & Baek, 2012; Endo et al, 2013; Gomes et al., 2013).

A patogenicidade dos *E. faecalis* depende não somente do seu número e diversidade de espécies presentes na infecção, mas também do potencial antigênico de seus mecanismos de virulência, que são capazes de perpetuar um processo inflamatório na região apical mesmo após a obturação do canal radicular. (Souza-Filho & Barbosa, 2003; Hahn & Liewehr, 2007; Ryu et al., 2009; Ozeki et al., 2015) Fatores de virulência são mecanismos que os microrganismos possuem para facilitar sua aderência, colonização, resistência, patogenicidade e evasão da resposta imune do hospedeiro (Medeiros et al., 2014). O papel dos fatores de virulência dos *Enterococcus* ainda não foi completamente elucidado e tem despertado interesse devido a sua habilidade em potencializar a infecção e de gerar respostas exacerbadas. Estas cepas, por estarem presas num ambiente nutricional restrito e seletivo podem possuir mecanismos de virulência diversificados e favorecer trocas genéticas entre elas (Wang et al., 2011; Camargo et al., 2014). A presença destes fatores de virulência no biofilme endodôntico pode ativar ou exacerbar respostas teciduais distintas na região periapical, por isso, torna-se imperioso entender o papel específico de cada um deles na patogenicidade do conteúdo infeccioso dos canais radiculares.

O ácido lipoteicóico (LTA) é um importante fator de virulência das bactérias Gram-positivas, dentre elas, *E. faecalis* e tem propriedades patogênicas similares ao lipopolissacárideo (LPS) de bactérias Gram-negativas (Ginsburg, 2002; Hermann et al., 2002; Han et al., 2003; Wang et al., 2003; Hahn e Liewehr, 2007; Siqueira Jr e Rocas, 2007; Baik et al., 2008; Ryu et al., 2009). O número de unidades de repetição e o teor de D-alanina (porção glicolipídica) parecem estar estreitamente relacionados com o seu potencial pró-inflamatório (Wang et al., 2003; Baik et al., 2008). No entanto, a estrutura química que dá ao LTA purificado a sua atividade biológica não foi completamnte elucidada. Esta molécula pode iniciar uma série de reações por ligação específica (CD14 e receptores toll-like 2) ou não específica (membrana de

fosfolípido; ativação do sistema do complemento; e liberação de citocinas pró-inflamatórias TNF-α, IL-1, IL-6, IL-8 e PGE_2) (Card, Jasuja e Gustafson, 1994; Ginsburg, 2002; Hermann et al., 2002; Costa, Souza-Filho e Barbosa, 2003; Han et al., 2003; Telles et al., 2003; Wang et al., 2003; Kayaoglu e Ørstavik, 2004; Hahn e Liewehr, 2007; Siqueira Jr e Rocas, 2007; Seo et al., 2008; Ryu et al., 2009). Sua difusão para os tecidos periapicais pode induzir o dano tecidual, tanto direta ou indiretamente (via sistema imune). Contudo, pouco se conhece a respeito da ação do LTA no desenvolvimento de sintomatologia dolorosa e reabsorção óssea.

Os fatores de virulência mais frequentemente relacionados com os Enterococcus são: *ace* (proteína de ligação ao colágeno), *asa* e *asa373* (substância de agregação), *cylA* (ativador de hemolisina), *efaA* (antígeno da endocardite), *esp* (proteína de superfície) e *gelE* (gelatinase). A expressão destes genes no biofilme endodôntico pode ativar ou exacerbar respostas teciduais distintas na região periapical, por isso, torna-se imperioso entender o papel específico de cada um deles na patogenicidade do conteúdo infeccioso dos canais radiculares (Sedgley et al., 2005; Wang et al., 2011; Endo et al., 2013).

Mais recentemente, estudos têm revelado um aumento surpreendente de cepas de *Enterococcus* spp resistentes a uma variedade de antibióticos comumente utilizados na terapêutica medicamentosa, principalmente pelo seu uso indiscriminado (Murray, 1990; Morrison *et al*., 1997; Poeschl et al., 2010; Skucaite et al., 2010; Endo et al., 2012, 2014). *Enterococcus* spp. têm adquirido determinantes genéticos que conferem resistência a várias classes de antibióticos, incluindo eritromicina, tetraciclina, cloranfenicol, e, mais recentemente, vancomicina (Murray, 1990; Morrison *et al*., 1997; Mundy *et al*., 2000; Embora a emergência de cepas resistentes seja mais pronunciada em infecções hospitalares ou sistêmicas (Poeschl et al., 2010), estudos de *Enterococcus* spp. possibilitam o monitoramento constante da sua resistência aos agentes antimicrobianos, permitindo a adoção de um tratamento mais eficaz (Skucaite et al., 2010).

A periodontite apical é um processo inflamatório dinâmico localizado na região periapical, com atuação de diferentes mediadores químicos produzidos por células do sistema imune em resposta ao estímulo causado pela ação dos microrganismos e seus bio-produtos. A exacerbação deste processo inflamatório, diretamente relacionada ao aumento da concentração desses mediadores, pode resultar na morte de tecidos e modular o aparecimento dos sinais e sintomas clínicos

e/ou radiográficos (Martinho et al., 2011; Endo et al., 2013, Zhao et al., 2013). A resposta do hospedeiro frente a agressões é caracterizada por um padrão no qual uma rede de mediadores químicos é acionada no intuito de debelar o processo inflamatório instalado na região periapical. LTA é capaz de estimular a liberação de citocinas inflamatórias, tais como IL1-α, IL1-β, IL6, IL10, TNF-α e PGE2 por diferentes linhagens celulares (Ryu et al., 2009), dentre as quais estão os macrófagos presentes em maior população, sendo considerados a principal fonte de produção de citocinas pró-inflamatórias (Martinho et al, 2010).

As citocinas atuam como mensageiros químicos no sistema imunológico, realizando comunicação com células de outros sistemas. A interpretação das mensagens fica a cargo da via metabólica a ser ativada naquele tipo de célula (Consolaro, 2009), podendo ser autócrina (atuando na mesma célula que a secreta), parácrina (em células diferentes) ou endócrina (agindo sistemicamente) (Tayal e Kalra, 1999). Suas principais funções são: mediar e regular respostas imunitárias, inflamação, hematopoiese e controle da proliferação e diferenciação celular. Durante o processo inflamatório há o recrutamento celular que é fator essencial para controlar a infecção estabelecida na região periapical. Isso é resultado da expressão de moléculas de adesão na superfície vascular de células endoteliais, induzidas por citocinas IL1-α, IL1-β, TNF-α e quimiocinas.

TNF-α é um dos principais mediadores da resposta inflamatória a bactérias Gram-positivas e outros microrganismos (Safavi et al., 1994; Ataoglu et al., 2002; Hong et al., 2004). Suas principais funções biológicas são o recrutamento de neutrófilos e monócitos para o local da infecção e a ativação destas células para erradicar os microrganismos. Já a IL1-β é capaz de induzir à síntese e à liberação de mediadores como a IL-6 e IL-8 e levar ao aparecimento de hipotensão, taquicardia, acidose láctica, neutrofilia, contudo, seu efeito biológico mais consistente é o aumento da síntese de prostaglandina E2 (PGE_2), que possui a capacidade de sensibilizar nociceptores (Van Deuren et al., 1992). O sinergismo existente entre o TNF-α e IL1-β é um fenômeno comumente descrito na literatura (Dinarello et al., 1986; Martinho et al., 2012). Claramente, ambas as citocinas são produzidas no mesmo sítio de inflamação e, desta forma, a interação de seus efeitos deve ser considerada exacerbada quando avaliada a severidade da doença.

Metaloproteinases da matriz (MMP) são enzimas são capazes de degradar todos os componentes da matriz extracellular (MEC) e proteínas da membrana

basal, atuando ativamente na remodelação óssea, seja em situações fisiológicas ou patológicas (Paula-Silva, da Silva e Kapila, 2010; Silva et al., 2012; Gomes et al., 2013; Sambandam e Neelakantan, 2014; Ozeki et al., 2015). Elas podem estar ancoradas à superfície celular ou serem secretadas por uma variedade de células, dentre elas leucócitos polimorfonucleares, monócitos, macrófagos, fibroblastos e osteoblastos (Ahmed et al., 2013).

Sua ativação está condicionada a uma regulação imunológica, que em condições desfavoráveis como a periodontite apical, fica na dependência de uma via de sinalização tecidual iniciada diretamente pelos microrganismos oriundos do canal radicular ou através dos seus fatores de virulência que ativam uma série de mediadores químicos, que podem promover a expressão das MMPs (Sambandam e Neelakantan, 2014).

Inibidores teciduais de MMPs (ITMMPs), citocinas, fatores de crescimento e indutor de metaloproteinases da matriz (EMMPRIN) são algumas moléculas regulatórias da expressão de MMPs (Hannas et al., 2007; Ozeki et al., 2015). Citocinas pró-inflamatórias como TNF-α e IL-1β que possuem maiores taxas de expressão em processos inflamatórios da região periapical podem modular a ação das MMPs, e consequentemente agir na destruição óssea ou até mesmo perpetuar uma inflamação periapical que não possibilite o reparo (Ozeki et al., 2014, 2015).

O papel dos microrganismos, seus bio-produtos e mediadores químicos da inflamação nas infecções endodônticas primárias já está bem elucidado na literatura científica (Martinho et al., 2010, 2012; Marinho et al., 2014, 2015). No entanto, nos casos onde houve insucesso do tratamento endodôntico, existe uma escassez de estudos que relacionam *in vivo* o real efeito do PQM e medicação intracanal (MIC) no controle da infecção e inflamação. A redução do tempo de contato da substância química auxiliar (SQA) e MIC e a simplificação das técnicas de instrumentação do canal radicular podem ser um dos fatores que influenciam na diminuição das taxas de sucesso. Por isso, torna-se importante o conhecimento e monitoramento dos fatores causais e mediadores químicos envolvidos neste processo, a fim de estabelecer estratégias de controle do conteúdo infeccioso dos canais radiculares (Baugh e Wallace, 2014; Gade et al., 2013, Miranda et al., 2013; Koçak et al., 2014; KungWani et al., 2014). Desta forma, este trabalho visa através do monitoramento microbiológico, dos níveis de LTA, citocinas pró-inflamatórias e MMPs, investigar *in*

vivo o efeito do PQM e da MIC na redução e/ou eliminação desse conteúdo em canais radiculares de dentes com periodontite apical pós-tratamento endodôntico.

PROPOSIÇÃO

Capítulo I

Caracterizar a microbiota Gram-positiva presente nas diferentes etapas da terapia endodôntica, e monitorar *in vivo* o efeito do PQM e da MIC na redução dos níveis de LTA e de bactérias cultiváveis em dentes com insucesso do tratamento endodôntico.

Capítulo II

Monitorar *in vivo* o efeito do PQM e da MIC na redução dos níveis das citocinas pró-inflamatórias (TNF-α e IL1-β) e das MMPs (-2, -3, -8, -9 e -13) em dentes com insucesso do tratamento endodôntico.

Capítulo III

Analisar a suscetibilidade antimicrobiana aos antibióticos freqüentemente prescritos na endodontia e determinar a prevalência dos fatores de virulência de cepas de *E. faecalis* isolados de dentes com insucesso do tratamento endodôntico.

CAPÍTULO I

Quantification of Lipoteichoic Acid Contents and Cultivable Bacteria at the Different Phases of Endodontic Therapy of Teeth with Post-Treatment Apical Periodontitis

Abstract

Aim: To quantify the levels of both LTA and cultivable bacteria at the different phases of the endodontic retreatment (ER) of teeth with post-treatment apical periodontitis. It also aimed to investigate the presence of Gram-positive microorganisms before and after chemomechanical preparation (CMP) and intracanal medication (ICM). **Methods**: Twenty infected root canals of single-rooted teeth were randomly assigned into two groups according to the irrigant used for CMP (n=10 *per* group): G1 - 2% chlorhexidine gel and G2 - 6% sodium hypochlorite. Root canal samples were taken using paper points before (S1) and after CMP (S2) and after 30 days of ICM with $Ca[OH]_2$ + 2% chlorhexidine gel (S3). Microorganisms were identified by culture technique using biochemical tests. Cultivable bacteria were determined by counting the colony-forming unit (CFU/mL). LTA levels were measured using the enzyme-linked immunosorbent assay (pg/mL). **Results**: A total of 70 Gram-positive bacteria, out of 102 bacteria isolated, were found in the root canals (54 in S1, 4 in S2 and 12 in S3). *Enterococcus faecalis* was the most frequent isolated bacteria in all phases of the ER. LTA (94.1±61.7) and cultivable bacteria (101.2±79.2) were present in all S1 samples. CMP decreased the overall levels of cultivable bacteria by 99.4% and LTA by 60.8% ($P< .05$), whereas the total overall reduction level of ICM on viable bacteria was 99.5% and on LTA was 76% ($P < 0.05$). CMP with 2% CHX gel (G1, 99.3%) was more effective ($P < 0.05$) than 6% NaOCl (G2, 92.1%). On the other hand, ICM showed 100% reduction in the CHX-group (G1) and 98.5% in NaOCl-group (G2). Regarding the reduction of LTA, CMP with 2% CHX gel (G1, 55.6%) was less effective ($P < 0.05$) than 6% NaOCl (G2, 67.5%). On the other hand, ICM showed 74.4% reduction in the CHX-group (G1) and 78.2% in NaOCl-group (G2) ($P > 0.05$).

Conclusion: Regardless the CMP and ICM the reduction rates of bacteria were higher compared to LTA. Gram-positive microorganisms were present in all phases of the endodontic retreatment.

Keywords: Bacteria. Chlorhexidine. Sodium hypochlorite. Enterococcus faecalis. Virulence factor.

Introduction

The role of microorganisms in the pathogenesis of apical periodontitis has been nicely elucidated by the literature (1-4) as they can perpetuate an infection after root canal filling or induce new inflammation in the periapical region (3,5-6).

The microbiota of teeth with failure of the endodontic treatment is predominantly composed of facultatively anaerobic microorganisms such as *Actinomyces, Candida* and *Enterococcus* species (7-8). *Enterococcus faecalis*, a facultative Gram-positive bacterium is the most detected microorganism using culture-dependent or independent techniques, being able to endure severe conditions of survival in the root canal with large variation of pH, temperature and O_2 tension (9-12). When exposed to environmental stress, some strains can adopt a viable existing state and resuscitate when normal conditions are re-established (13). Even after bacterial death, some components of the Gram-positive bacteria cell wall (e. g. LTA) persist in the root canal for long periods of time, which can cause chronic inflammation (6,11,14-16).

The antigenic complexity of the root canal content is influenced by the diversity and the number of microbial species, including synergistic and antagonistic relationships and presence of bacterial virulence factors (e.g. LTA), modulating the toxicity of this infectious content (17-18).

Lipoteichoic acid (LTA) is an important virulence factor of the Gram-positive bacteria (9,19-22) which is released during bacterial multiplication, mainly after bacteriolysis by lysozyme, bactericidal cationic peptides, phospholipase A, cathepsins or beta-lactam antibiotics (19). It has pathogenic properties similar to the lipopolysaccharides (LPS) of Gram-negative bacteria (3,16,19,21-23), resulting in well-known injuries to dental pulp and periapical tissues. However, LTA differs from LPS in term of structure and gene transcription (22).

LTA can begin a series of reactions by binding to specific receptors (CD14 and toll-like 2 receptors) or by non-specific events (phospholipid membrane; complement system activation; release of pro-inflammatory cytokines TNF-α, IL-1β, IL-6 IL-8 and PGE_2; angiogenesis regulation; release of hydrolases, proteases, prostaglandins and reactive oxygen species from neutrophils and macrophages; and regulation, recruitment and activation of neutrophils) (3,15-16,19-24).

Thus, the present study aimed to investigate the levels of LTA and the cultivable Gram-positive microorganisms and evaluate the effect of chemomechanical preparation (CMP) and intracanal medication (ICM) on the reduction of LTA and bacteria of teeth with post-treatment apical periodontitis.

Materials and Methods

Patient Selection

Twenty patients were selected from those who attended the Piracicaba Dental School, State University of Campinas- UNICAMP, Piracicaba, SP, Brazil, with a need for nonsurgical endodontic retreatment.

The Human Volunteers Research and Ethics Committee of the Piracicaba Dental School approved a protocol (# 018/2014), describing the specimen collection for this investigation, and all patients signed an informed consent for their participation in this research. The age of the patients ranged from 30 to 60 years. All selected teeth (n=20) had been previously single root-filled and showed radiographic evidence of apical periodontitis.

Failure of root canal treatment was determined based on clinical and radiographic examinations. Presence of persistent periapical radiolucent lesion, voids in or around the root canal filling, persistent symptoms such as pain of palpation, discomfort to percussion, persistent sinus were considered reasons for retreatment (7).

Exclusion criteria were as follows. Subjects who had received antibiotic treatment within the preceding three months; reported systemic disease starting with ASA 3 (American Society of Anestesiology); teeth that could not be isolated with rubber dam, teeth with absence of coronary sealing, and teeth with periodontal pockets deeper than 3 mm were excluded.

Endodontic sample collection and clinical procedures

The teeth were isolated with rubber dam. The crown and surrounding structures were disinfected with 30% hydrogen peroxide (volume/volume for 30 seconds) followed by 2.5% sodium hypochlorite (NaOCl) for the same period of time

and then inactivated with 5% sodium thiosulfate (25). Disinfection of the external surfaces of the crown was checked by taking a swab sample from the crown surface and streaking it onto blood agar plates, which were then incubated aerobically and anaerobically.

The sampling procedures were performed according to Martinho and Gomes (25). Under anesthesia (2% lidocaine with 1:100,000 epinephrine), a two-stage access preparation was performed. The access cavity was made without the use of water spray but under manual irrigation with sterile saline and by using sterile high-speed diamond bur. This first stage was performed to promote a major removal of contaminants. In the second stage, before entering the pulp chamber, the access cavity was disinfected according to the decontamination protocol described above. Disinfection of the internal surface of the access cavity was checked as previously described, and all procedures were performed aseptically. Root-filling materials were removed by using Reciproc R25 files (VDW, Munich, Germany) in the working length obtained by preoperatory radiography and used according to the manufacturer's instructions in a crown-down technique, with no chemical solvent.

Before the first sample (S1) of the root canal, a K-file #20 (Dentsply Maillefer, Ballaigues, Switzerland) was used to confirm the working length (previously estimated by radiographs), with an apex locator (Novapex; Forum Technologies, Rishon le-Zion, Israel). A sterile paper point (Dentsply-Maillefer, Ballaigues, Switzerland) was then introduced into the full length of the canal and retained in position during 60 seconds for LTA sampling. Next, this paper point was placed in a sterile tube for enzyme-linked immunosorbent assay (ELISA). Other three paper points were pooled in a sterile tube containing 1 mL of VMGA Ⅲ transport medium (26) for microbial sampling. The samples were transported within 15 minutes to an anaerobic workstation (Don Whitley Scientific, Bradford, UK) for bacterial culture analysis. The LTA samples were frozen at −80 °C for further analysis.

Root canals were then prepared by using Reciproc R40 files (VDW, Munich, Germany) according to the manufacturer's instructions in a reciprocating working motion generated by the motor. The instrument was used in an in-an-out pecking motion of about 3 mm in amplitude with apical pressure. After three pecking motions, the instrument was removed from the canal and cleaned. Next, a K-file #20 was taken to the working length (WL) to check whether the canal was patent. These

procedures were repeated until the Reciproc instrument reached the WL (zero point displayed on the apex locator).

Retreatment was deemed complete when the Reciproc R40 file reached the working length, with no filling material covering the instrument and canal walls being smooth and free of visible debris. Furthermore, a close inspection under high magnification with dental operating microscope (DF Vasconcelos S.A., São Paulo, SP, Brazil) showed complete removal of gutta-percha.

The twenty infected root canals of single-rooted teeth with post-treatment apical periodontitis were divided randomly into two groups according to the chemical substances used.

• Group 1 (n = 10): 2% chlorhexidine gel (CHX)
• Group 2 (n = 10): 6% sodium hypochlorite (NaOCl)

Calcium hydroxide + 2% cholorhexidine gel was used as ICM in all cases for 30 days.

EndoVac System (Discus Dental, Culver, CA, USA) was used to irrigate both groups, using saline in group 1 and NaOCl in group 2. In group 1, during instrumentation the root canals were filled with 1 mL of 2% CHX gel (Endogel; Itapetininga, SP, Brazil) using a syringe (27-gauge needle) before the use of each instrument and immediately rinsed afterwards with 5 mL of saline solution using the EndoVac System. In the end of the instrumentation, CHX was inactivated with 5 mL of 5% Tween-80 and 0.07% (w/v) lecithin solution during 1-minute period, which was removed with 5 mL of saline solution

In group 2 during instrumentation the root canals were filled with 1 mL of 6% NaOCl (Drogal; Piracicaba, SP, Brazil), using a syringe (27-gauge needle) before the use of each instrument and immediately rinsed afterwards with 5 mL of 6% NaOCl using the EndoVac System. In the end of the instrumentation, NaOCl was inactivated with 5 mL of a solution of 5% sodium thiosulfate (Drogal, Piracicaba, SP, Brazil) for 60 seconds, which was also removed with 5 mL saline solution.

Before the second sampling procedure (S2), a rinse with 5 mL of 17 % EDTA was applied continuously for 3 minutes under stirring with ultrasound (Advanced SE, Microdont, São Paulo, SP, Brazil) with tip ET40 (Satelec / Acteon, Mount Laurel, NJ, USA), for 60 seconds alternately followed by a final rinse with 5 mL of sterile saline

solution. Next, a second LTA and microbiological samples were taken (S2) as previously described.

The canal was dried with paper points. A calcium hydroxide [$Ca(OH)_2$] paste was placed over the entire length of the prepared canal by using Lentulo spiral fillers. The access cavity was then temporarily sealed with a temporary cement (Coltosol, Coltène/Whaledent, Cuyahoga Falls, OH, USA) at a thickness of at least 2 mm, and a second layer of composite material (Filtek Z250; 3M ESPE, St. Paul, MN, USA) was applied in combination with a single bond adhesive (3M ESPE). After 30 days, the canal was aseptically accessed and the medication removed with 5 mL saline solution and the master apical file (# 40). $Ca(OH)_2$ activity was neutralized with 0.5% citric acid during 1-minute period, which was then removed with 5 mL of saline solution. Next, the third sample (S3) was immediately taken.

A final irrigation with 3 ml of 17% EDTA with ultrasound as described previously, followed by the irrigation with 5 ml of sterile saline. Finally, as all canals were asymptomatic and dried, the teeth were filled with a single Reciproc gutta-percha cone and Endométhasone sealer (Septodont, Saint-Maur-des-Fossés, France). Access cavities were restored with Coltosol (Coltène/Whaledent) at a thickness of at least 2 mm and a second layer of composite material (Filtek Z250; 3M ESPE) was applied in combination with single bond adhesive.

Culture Procedure and Microbial Identification

The method for counting the colony-forming unit (CFU) has been previously published by Martinho and Gomes (25). Briefly, the tubes containing root canal samples were shaken thoroughly for 60 seconds (Vortex; Marconi, Piracicaba, São Paulo, Brazil) and then serial 10-fold dilutions were made to 10^{-4} in tubes containing Fastidious Anaerobe Broth (FAB; Lab M, Bury, UK). By using sterile plastic spreaders, 50µL of each serial dilution were plated onto 5% defibrinated sheep blood Fastidious Anaerobe Agar (FAA; LAB M, Bury, UK) to obtain non-selectively obligate anaerobes and facultative anaerobes. The plates were incubated at 37°C in anaerobic atmosphere for up to 14 days. After this period, colony-forming units (CFUs) were visually quantified for each plate.

The microbial identification was performed from each bacterial plate, where representative colonies of each morphologic type were cultured. Pure cultures were initially characterized according to their gaseous requirements, Gram-stain

characteristic, and ability to produce catalysis. They were then biochemically identified using specific kits (API 20 A, API 20 Strep, API 20 Staph, API NH, BioMérieux, Marcy-l'Etoile, France).

Measurement of LTA Levels

Levels of LTA in the different phases of endodontic retreatment were measured by using the human lipoteichoic acid ELISA Kit (My BioSource; San Diego, CA, USA). Standard, control, and sample solutions were added to an ELISA well plate, which had been precoated with the specific monoclonal antibody for LTA supplied by the manufacturer. Anti-LTA antibodies labeled with biotin were added to unite with streptavidin-HRP, forming an immune complex. The plate was incubated for 60 minutes at 37°C and then washed for removal of unbound enzymes. A substrate solution containing hydrogen peroxidase and chromogen was added and allowed to react. Shades of solution and the concentration of LTA were positively correlated. The levels of LTA were assessed by an ELISA reader at 450 nm and normalized for negative control values. Each densitometric value, expressed as mean and standard deviation, was obtained from two independent experiments.

Statistical Analysis

Data collected for CFUs and LTA concentrations were statistically analyzed by using SAS for Windows (SAS Inc, Cary, NC, USA). The normality of the data was verified by the Shapiro-Wilk test, and those presenting normal distribution were analyzed with one-way analysis of variance and *post hoc* Tukey-Kramer method for inter-group analysis. Paired t-test and repeated measure analysis of variance were also applied for intra-group analysis at the different phases of endodontic therapy. All tests were performed at significance of 5%.

Results

The samples obtained from the external surfaces of the selected teeth before and after pulp chamber penetration presented no microbial growth, thus showing that the whole procedure was performed under sterile conditions.

A total of 70 Gram-positive bacteria, out of 102 bacteria isolated, were found in the root canals (54 in S1, 4 in S2 and 12 in S3). The most prevalent Gram-positive

genera found in the initial samples (S1) were *Actinomyces, Aerococcus, Enterococcus, Gemella* and *Staphylococcus*. After CMP, the most frequently-detected genera were *Enterococcus* and *Micrococcus*. After ICM the genera most frequently detected were *Enterococcus* and *Staphylococcus* (Figure 1). *Enterococcus faecalis* was the most frequently found bacteria in all phases of the endodontic retreatment (Figure 1).

Cultivable bacteria (101.2±79.2) and LTA (94.1±61.7) were present in all S1 samples (20/20). CMP decreased the overall levels of cultivable bacteria by 99.4% ($P < 0.05$) and of LTA by 60.8% ($P < 0.05$), whereas the total overall reduction level of ICM on viable bacteria was 99.5% and on LTA was 76% ($P < 0.05$). Figure 2 shows the reduction of cultivable bacteria and lipoteichoic acid at the different phases of endodontic retreatment.

Regarding the reduction of cultivable bacteria, CMP with 2% CHX gel (G1, 99.3%) was more effective ($P < 0.05$) than 6% NaOCl (G2, 92.1%). On the other hand, ICM showed 100% reduction in the CHX-group (G1) and 98.5% in NaOCl-group (G2).

Regarding the reduction of LTA, CMP with 2% CHX gel (G1, 55.6%) was less effective ($P < 0.05$) than 6% NaOCl (G2, 67.5%). On the other hand, ICM showed 74.4% reduction in the CHX-group (G1) and 78.2% in NaOCl-group (G2), without statistical difference between the groups after the use of ICM ($P > 0.05$).

It is important to stress that the reduction of cultivable bacteria was greater than the reduction of LTA in all phases of ER (Table 1).

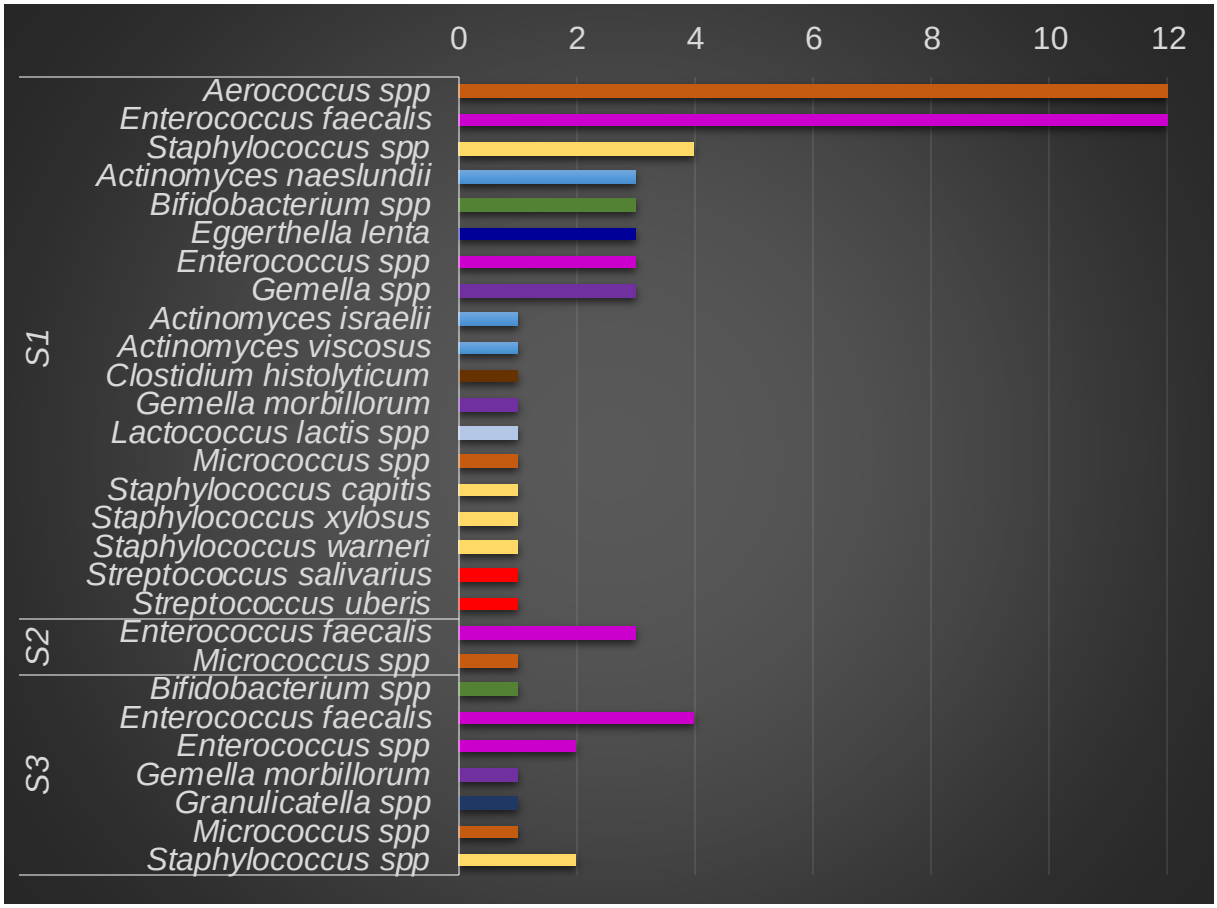

Figure 1. Gram-positive found in microbial culture at the different phases of root canal therapy with pos-treatment apical periodontitis.

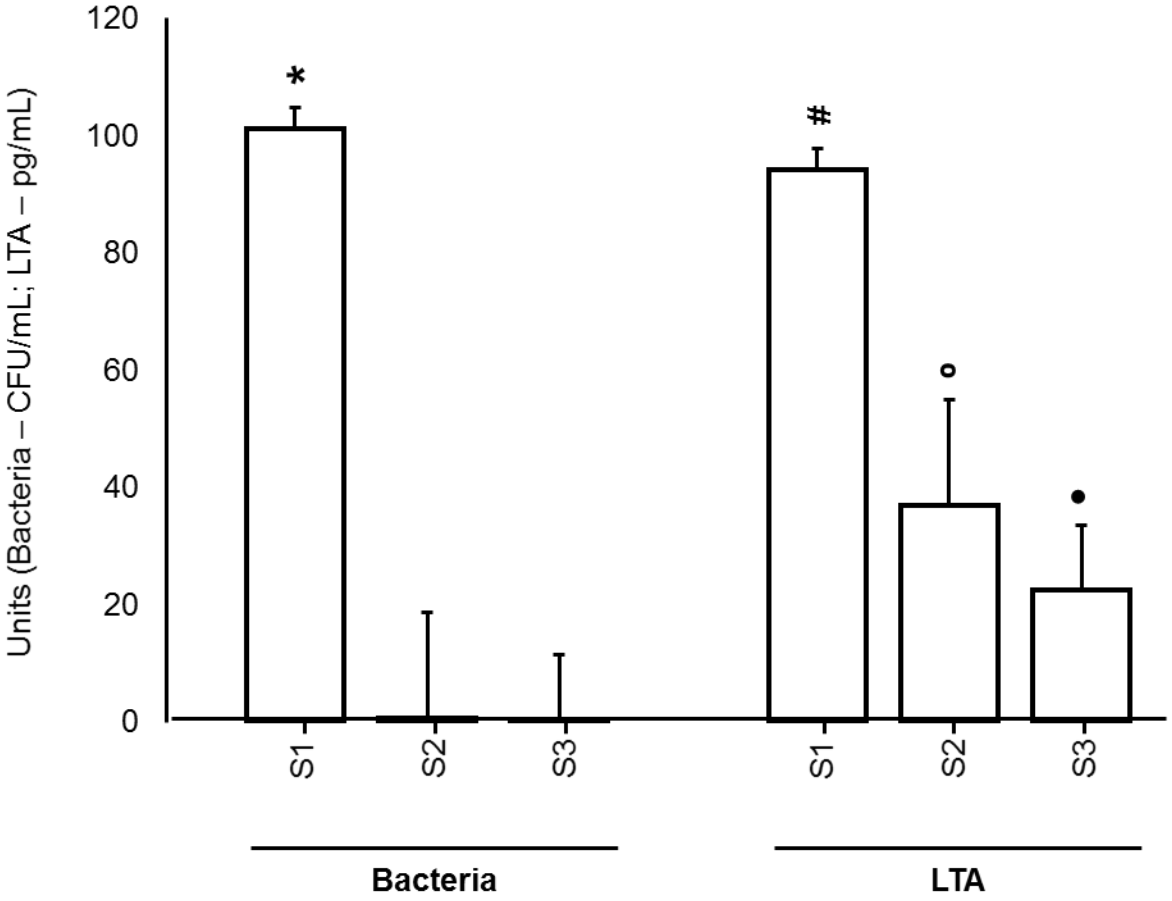

Figure 2. Reduction of cultivable bacteria and lipoteichoic acid at the different phases of endodontic therapy of teeth with post-treatment apical periodontitis.

Table 1 – Cultivable bacterial (x10^2 CFU/mL), LTA concentration (pg/mL), standard deviation and reduction (%) found in the 20 cases of post-treatment apical periodontitis.

Treatment phase	Overall values	Overall reduction (%)	**Chemical substance**			
			2% Chlorhexidine gel	Reduction (%)	6% sodium hypochlorite	Reduction (%)
Bacterial						
S1	101.2±79.2 [a]	-	129.10±83.4 [a]	-	73.2±67.4 [a]	-
S2	0.6±1.4 [b]	99.4	0.90±2.8 [b]	99.3	5.8±6.1 [c]	92.1
S3	0.5±0.8 [b]	99.5	0.0±0.0 [b]	100	1.1±1.9 [b]	98.5
LTA						
S1	94.1±61.7 [a]	-	107.2±77.7 [a]	-	80.9±45.3 [a]	-
S2	36.9±28.8 [b]	60.8	47.6±33.6 [b]	55.6	26.3±21.4 [c]	67.5
S3	22.5±14.5 [c]	76	27.4±16.9 [c]	74.4	17.6±11.3 [c]	78.2

Note: CFU, colony-forming units; CMP, chemomechanical preparation; ICM, intracanal medication; LTA, lipotecichoic acid. Columns and row with different letters indicate statistical differences ($P< 0.05$).

Discussion

Our results confirm the strong presence of Gram-positive bacteria in infections related to the failure of endodontic treatment as 70/102 microorganisms were found in the initial samples of the root canals investigated, agreeing with the findings of the literature (4,7,12). Moreover, *Enterococcus faecalis* was the most prevalent bacteria involved in this clinical situation (4,8). This bacterium has been associated with the ability to remain inside the root canal even after the endodontic procedures (27).

The literature shows that elimination of bacteria from root canals after CMP ranges between 80 and 95% (8, 28-31). In the present study, CMP with either CHX or NaOCl was able to reduce the bacterial levels in 99.3% and 92.1% respectively. The group irrigated with CHX achieved a greater reduction on the bacterial levels after CMP, probably because CHX has the ability to act on both Gram-positive bacteria and Gram-negative bacteria (12). The total overall reduction of ICM on the bacterial levels was 99.5%. ICM was more effective in reducing the bacterial levels in root canals irrigated with CHX (100%).

Regarding the LTA reduction, after CMP, the group irrigated with NaOCl achieved a greater reduction on the LTA levels (67.5%) compared to the CHX group (55.6%). The use of intracanal medication was able to reduce even more the LTA levels (G1: 74.4%; G2: 78.2%).

It is important to mention that the greatest reduction rate of microorganisms and LTA was achieved after CMP. The intracanal medication actually reduced 16.7% (microorganisms) and 39% (LTA). However, considering the cumulative effect of all phases it came to 99.5% and 76%, respectively.

Our findings that CMP and ICM are more effective in reducing microbial levels than LTA levels are similar to the LPS reduction profile either in primary endodontic infection (17,34-35) or in secondary endodontic infection (8). In primary infections the LPS reduction percentual after CMP is between 59%-98.06% (35, 36) and in secondary infections in 60.6% (8), which is in agreement with the present study.

High levels of bacteria and LTA may be responsible for the maintenance of an inflammatory process in the periapical region and consequent failure of endodontic treatment (9,22). Further studies are needed to test the acceptable clinical levels of residual LTA and also to confirm the results found in the present work, as there is a

lack in the literature regarding clinical studies investigating the presence of LTA in infected root canals.

It was concluded that regardless the CM and ICM the reduction rates of bacteria were higher compared to LTA. Gram-positive microorganisms were present in all phases of the endodontic retreatment.

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The authors deny any conflicts of interest related to this study.

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CAPÍTULO II

Effectiveness of root canal procedures on the reduction proinflammatory cytokines and matrix metalloproteinases in cases of post-treatment apical periodontitis

Abstract

Aim: To monitor *in vivo* the effect of chemomechanical preparation (CMP) and intracanal medication (ICM) on the reduction of proinflammatory cytokines (PIC) (TNF-α and IL1-β) and matrix metalloproteinases (MMPs) (-2, -3, -8, -9 and -13) in root canals of teeth with post-treatment apical periodontitis. **Methodology:** Twenty infected root canals of teeth single-rooted were randomly assigned into two groups according to the irrigant used for CMP (n = 10 *per* group): G1 - 2% chlorhexidine gel and G2 - 6% sodium hypochlorite. Root canal contents were taken by using paper points before (S1) and after CMP (S2) and after 30 days of ICM ($Ca[OH]_2$ + 2% chlorhexidine gel) (S3). PIC and MMP (pg/mL) were measured using enzyme-linked immunosorbent assay. **Results:** PIC and MMP were present in all S1 samples. Lower initial values were found for PIC (TNF-α: 8.8±4.7; IL1-β: 1.2±0.4) compared to the levels of MMP-2 (803.7±96.4), followed by -3 (453.9±229.3), -8 (245.9±122.4), -9 (129.4±29.6) and -13 (70.8±12.8). Decreased levels of PIC and MMP were found for all groups ($P < 0.05$) in all S2 samples. On the other hand, PIC, MMP-2 and MMP-13 increased after the ICM ($P < 0.05$). There was no difference between the chemical substances tested on PIC reduction in S2 ($P > 0.05$). Regarding MMP, 2% CHX gel reduced the levels of all of them in S2 ($P < 0.05$), whereas 6% NaOCl reduced only MMP-2, -3 and -13 ($P < 0.05$). TNF-α and IL1-β increased in S3 for both G1 and G2 ($P < 0.05$). The ICM reduced MMP-3 and -8 only in G2 ($P < 0.05$). However, there was an increase of MMP in S3 ($P < 0.05$) for G1 (-3, -8, -9 and -13) and G2 (-2 and -13). **Conclusion:** regardless of the chemical substance tested, CMP is effective in reducing PIC and MMP. While the ICM increases the levels of TNF-α, IL-1β, MMP-2 e MMP-13.

Keywords: Bacteria. Chlorhexidine. Sodium hypochlorite. Enterococcus faecalis. Cytokines. Matrix metalloproteinases.

Introduction

The establishment of microorganisms and their by-products inside the root canals is one of the main factors related to failure of endodontic treatment, which is characterized by the persistence or emergence of apical periodontitis after root canal filling (Gomes et al. 1996, Pinheiro et al. 2003ab, Zhang et al. 2012, Rahimi et al. 2014, Siqueira Jr et al. 2014). As periodontitis is a dynamic inflammatory process located at the periapical region, the type of immune-inflammatory response is determined by a network of chemical mediators produced by immune cells in response to the stimulius caused by the action of microorganisms and/or virulence factors, which may result in death of tissues and modulate the appearance of clinical and/or radiographic signs and symptoms (Martinho et al. 2011, Endo et al. 2013, Zhao et al. 2013).

In infected root canals, high concentrations of tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL1-β) can promote the tissue destruction (Hong et al. 2004, Martinho et al. 2011), mainly by stimulating macrophages to produce matrix metalloproteinases (MMP), causing destruction of the extracellular matrix (ECM) (Pezelj-Ribaric et al. 2007). The destruction of the periapical tissues mediated by TNF-α and IL1-β appears to be directly related with high levels of infectious content present in the root canal (Martinho et al. 2012). TNF-α and IL1-β have been frequently detected in teeth with periapical lesions, root canals with exudate (Barkhordar et al. 1992, Matsuo et al. 1994, Martinho et al. 2010). Particularly high levels of IL1-β appear to be related to the presence of clinical signs/symptoms (Lim et al. 1994) and periapical bone destruction (Matsuo et al. 1994, Ataoglu et al. 2002). However, the literature is scanty regarding the role of pro inflammatory cytokines (PIC) in disease processes of periapex, especially in cases related to the failure of endodontic treatment.

MMPs belong to a family of enzymes known as to be zinc-dependent endopeptidases and are able to degrade ECM components, such as collagen, proteoglycans, fibronectin, laminin and elastin. Synthesized as pro-enzymes, most MMPs are secreted before conversion to their active form from gradual mechanisms (Ahmed et al. 2013, Sambandam & Neelakantan 2014). Additional MMP substrates include cytokines, chemokines, growth factors and binding proteins, cell/cell adhesion

molecules, and other proteinases. Their classification is based on the substrate specificity: collagenases, gelatinases, estromelysins, matrilysins, membrane-type MMP and others (Sambandam & Neelakantan 2014, Ozeki et al. 2014, Ozeki et al. 2015, Hannas et al. 2007, Gomes et al. 2013). Under physiological conditions, the synthesis and activity of MMPs are severely controlled by the body, because the increase in their levels may threaten the integrity of the cells and cause apoptosis (Ozeki et al. 2014, Ozeki et al. 2015). This instability occurs routinely in pathological situations such as inflammation and tissue invasion (Hannas et al. 2007).

Knowing the role of PIC and MMPs in modulating tissue displacement in periapical pathologies may contribute to the understanding and upper interpretation of clinical manifestations of apical periodontitis. The reduction of PIC and MMP levels by chemomechanical preparation (CMP) and intracanal medication (ICM) in teeth with apical periodontitis is a challenge, since it could enable immune balance and tissue healing. Therefore, the aim of this study was to monitor *in vivo* the effect of both CMP and ICM on the reduction of PIC and MMPs in root canals of teeth with post-treatment apical periodontitis.

Materials and Methods

Patient Selection

Twenty patients were selected from those who attended the Piracicaba Dental School, State Univesity of Campinas- UNICAMP, Piracicaba, SP, Brazil, with a need for nonsurgical endodontic retreatment.

The Human Volunteers Research and Ethics Committee of the Piracicaba Dental School approved a protocol (# 018/2014), describing the specimen collection for this investigation, and all patients signed an informed consent for their participation in this research. The age of the patients ranged from 30 to 60 years. All selected teeth had been previously single root-filled and showed radiographic evidence of apical periodontitis.

Failure of root canal treatment was determined based on clinical and radiographic examinations. Presence of persistent periapical radiolucent lesion, voids in or around the root canal filling, persistent symptoms such as pain of palpation, discomfort to percussion, persistent sinus were considered reasons for retreatment (Pinheiro et al. 2003ab).

Exclusion criteria were as follows. Subjects who had received antibiotic treatment within the preceding three months; reported systemic disease starting with ASA 3 (American Society of Anestesiology); teeth that could not be isolated with rubber dam, teeth with absence of coronary sealing, and teeth with periodontal pockets deeper than 3 mm were excluded.

Endodontic sample collection and clinical procedures

The teeth were isolated with rubber dam. The crown and surrounding structures were disinfected with 30% hydrogen peroxide (volume/volume for 30 seconds) followed by 2.5% sodium hypochlorite (NaOCl) for the same period of time and then inactivated with 5% sodium thiosulfate (Martinho & Gomes 2008). Disinfection of the external surfaces of the crown was checked by taking a swab sample from the crown surface and streaking it onto blood agar plates, which were then incubated aerobically and anaerobically.

The sampling procedures were performed according to Martinho & Gomes (2008). Under anesthesia (2% lidocaine with 1:100,000 epinephrine), a two-stage access preparation was performed. The access cavity was made without the use of water spray but under manual irrigation with sterile saline and by using sterile high-speed diamond bur. This first stage was performed to promote a major removal of contaminants. In the second stage, before entering the pulp chamber, the access cavity was disinfected according to the decontamination protocol described above. Disinfection of the internal surface of the access cavity was checked as previously described, and all procedures were performed aseptically. Root-filling materials were removed by using Reciproc R25 files (VDW, Munich, Germany) in the working lenght obtained by preoperatory radiography and used according to the manufacturer's instructions in a crown-down technique, with no chemical solvent.

Before the first sample (S1) of the root canal, a K-file #20 (Dentsply Maillefer, Ballaigues, Switzerland) was used to confirm the working length (previously estimated by radiographs), with an apex locator (Novapex; Forum Technologies, Rishon le-Zion, Israel). A sterile paper point (Dentsply-Maillefer, Ballaigues, Switzerland) was then introduced 1 mm over the apex reaching the periapical tissues and maintained in position during for 60 seconds. Next, the paper point was placed in

a sterile tube for enzyme-linked immunosorbent assay (ELISA) and then frozen at −80 °C for further analysis.

Root canals were then prepared by using Reciproc R40 files (VDW, Munich, Germany) according to the manufacturer's instructions in a reciprocating working motion generated by the motor. The instrument was used in an in-an-out pecking motion of about 3 mm in amplitude with apical pressure. After three pecking motions, the instrument was removed from the canal and cleaned. Next, a K-file #20 was taken to the working length (WL) to check whether the canal was patent. These procedures were repeated until the Reciproc instrument reached the WL (zero point displayed on the apex locator).

Retreatment was deemed complete when the Reciproc file reached the working length, with no filling material covering the instrument and canal walls being smooth and free of visible debris. Furthermore, a close inspection under high magnification with dental operating microscope (DF Vasconcelos S/A, São Paulo, Brazil) showed complete removal of gutta-percha.

The twenty infected root canals of single-rooted teeth with post-treatment apical periodontitis were divided randomly into two groups according to the chemical substances used.

- Group 1 (n = 10): 2% chlorhexidine gel (CHX)
- Group 2 (n = 10): 6% sodium hypochlorite (NaOCl)

Calcium hydroxide + 2% cholorhexidine gel was used as intracanal medication (ICM) in all cases for 30 days.

EndoVac System (Discus Dental, Culver, CA, USA) was used to irrigate both groups, using saline in group 1 and NaOCl in group 2. In group 1, during instrumentation the root canals were filled with 1 mL of 2% CHX gel (Endogel; Itapetininga, SP, Brazil) using a syringe (27-gauge needle) before the use of each instrument and immediately rinsed afterwards with 5 mL of saline solution using the EndoVac System. In the end of the instrumentation, CHX was inactivated with 5 mL of 5% Tween-80 and 0.07% (w/v) lecithin solution during 1-minute period, which was removed with 5 mL of saline solution

In group 2 during instrumentation the root canals were filled with 1 mL of 6% NaOCl (Drogal; Piracicaba, SP, Brazil), using a syringe (27-gauge needle) before

the use of each instrument and immediately rinsed afterwards with 5 mL of 6% NaOCl using the EndoVac System. In the end of the instrumentation, NaOCl was inactivated with 5 mL of a solution of 5% sodium thiosulfate (Drogal, Piracicaba, SP, Brazil) for 60 seconds, which was also removed with 5 mL saline solution.

Before the second sampling procedure (S2), a rinse with 5 mL of 17 % EDTA was applied continuously for 3 minutes under stirring with ultrasound (Advanced SE, Microdont, São Paulo, SP, Brazil) with tip ET40 (Satelec / Acteon, Mount Laurel, NJ), for 60 seconds alternately followed by a final rinse with 5 mL of sterile saline solution. Next, a second paper point was introduced 1 mm over the apex reaching the periapical tissues and maintained in position during for 60 seconds. Following, the paper point was placed in a sterile tube for enzyme-linked immunosorbent assay (ELISA) and then frozen at −80 °C for further analysis.

The canal was dried with paper points. A calcium hydroxide [$Ca(OH)_2$] paste was placed over the entire length of the prepared canal by using Lentulo spiral fillers. The access cavity was then temporarily sealed with a temporary cement (Coltosol, Coltène/Whaledent, OH, USA) at a thickness of at least 2 mm, and a second layer of composite material (Filtek Z250; 3M ESPE, St. Paul, MN) was applied in combination with a single bond adhesive (3M ESPE). After 30 days, the canal was aseptically accessed and the medication removed with 5 mL saline solution and the master apical file (# 40). $Ca(OH)_2$ activity was neutralized with 0.5% citric acid during 1-minute period, which was then removed with 5 mL of saline solution. Next, the third sample (S3) was immediately taken, as mentioned before.

The canals were then irrigated with 3 mL of 17% EDTA using the ultrasound as previously described, followed by the irrigation with 5 mL of sterile saline. Finally, as all the canals were asymptomatic and dried, the teeth were filled with a single Reciproc gutta-percha cone and Endométhasone sealer (Septodont, Saint-Maur-des-Fossés, France). Access cavities were restored with Coltosol (Coltène/Whaledent) at a thickness of at least 2 mm and a second layer of composite material (Filtek Z250; 3M ESPE) was applied in combination with single bond adhesive.

Measurements of PIC and MMP Levels

Levels of TNF-α, IL1-β, MMP-2, MMP-3, MMP-8, MMP-9 and MMP-13 in the different phases of the endodontic retreatment were measured with the specific Quantikine® Human ELISA Kit (R&D Systems®; Minneapolis, MN, USA) using the

quantitative sandwich enzyme immunoassay technique. Next, standard, control, or sample solution was added to an ELISA well plate, which had been precoated with specific immobilized monoclonal antibody supplied by the manufacturer for each molecule to be tested. After washing away the unbound substances, an enzyme-linked polyclonal antibody specific for each molecule was added to the wells, forming an immune complex. The plate was incubated for 60 minutes at room temperature on the shaker before washing for removal of unbound enzymes. After another washing, a substrate solution containing hydrogen peroxidase and chromogen was added and allowed to react. The color development was stopped and the intensity of the color was measured, thus revealing the concentration of PIC and MMPs. The levels of PIC and MMPs were assessed by an ELISA reader at 450 nm and the negative control values were normalized. Each densitometric value, expressed as mean and standard deviation, was obtained from two independent experiments.

Statistical Analysis

The data collected for PIC and MMP concentrations were statistically analyzed by using SAS for Windows (SAS Inc, Cary, NC, USA). The normality of the data was verified by Shapiro-Wilk test, with those presenting normal distribution being analyzed with *post-hoc* one-way analysis of variance and Tukey-Kramer method for intra-group analysis at the different phases of endodontic therapy. In all statistical tests, the significance value was set at 5%.

Results

PIC and MMPs were present in all S1 samples with varying levels between them. Overall, it was observed a decrease in the levels of PIC and MMPs after CMP in all groups ($P < 0.05$), whereas ICM was not effective in reducing the levels of MMPs ($P > 0.05$). A statistically significant increase of TNF-α, IL-1-β and MMP-2, was observed in S3 (ICM).

In S2 (CMP) it was found a reduction of PICI levels of TNF-α (89.85%) and IL-1β (89.33%) ($P < 0.05$), with no difference between the groups ($P > 0.05$). However, regarding the MMPs reduction, it was found that 2% CHX gel (G1) reduced the levels

of all metalloproteinases in S2 ($P < 0.05$), whereas 6% NaOCl (G2) was effective in the reduction of MMP-2, MMP-13 and MMP-3 ($P < 0.05$).

In S3, there was a significant increase of PIC after the use of ICM ($P < 0.05$). Regarding MMPs, ICM showed reduction only for MMP-3 e MMP-8 in G2 ($P < 0.05$). There was a significant increase of MMPs ($P < 0.05$) in S3 in both G1 (MMP-3, MMP-8, MMP-9 and MMP-13) and G2 (MMP-2 and MMP-13).

In general, CMP was effective in reducing PIC and MMPs, whereas ICM showed no similar action in comparison with CMP. Means, standard deviations and percentages of the overall reduction and for each chemical substance in the different phases of endodontic therapy are listed in Table 1.

Table 1 – PIC and MMps (pg/mL) concentrations, standard deviation and percentage reduction achieved by the use of different irrigants and intracanal medication during endodontic therapy of teeth with post-treatment apical periodontitis.

	Treatment phase	Overall	Reduction (%)	**Chemical substance**			
				2% Chlorhexidine gel	Reduction (%)	6% Sodium hypochlorite	Reduction (%)
TNF-α	S1	8,8±4,7 [a]	---	7,8± 3,6 [a]	---	9,7±5,6 [a]	---
	S2	0,9±0,9 [b]	89,7	0,9±1 [b]	88,5	0,9±0,9 [b]	90,7
	S3	3,3±4,1 [c]	-266,6	2,9±2,8 [c]	-222,2	3,6±5,2 [c]	-300
IL1-β	S1	1,2±0,4 [a]	---	1,2±0,5 [a]	---	1,1±0,3 [a]	---
	S2	0,1±0,1 [b]	91,6	0,1±0,1 [b]	91,6	0,1±0,1 [b]	91,6
	S3	0,7±0,2 [c]	-600	0,7±0,3 [c]	-600	0,7±0,2 [c]	-600
MMP-2	S1	803,7±96,4 [a]	---	843,0±119,0 [a]	---	764,4±36,6 [a]	---
	S2	739,6±58,3 [b]	7,8	750,1±67,7 [b]	11	729,0±44,7 [b]	4,6
	S3	758,4±64,1 [c]	-2,5	764,0±79,2 [b]	-1,8	752,7±43,3 [a]	-3,2
MMP-3	S1	453,9±229,3 [a]	---	511,7±247,9 [a]	---	396,1±192,4 [a]	---
	S2	316,2±142,3 [b]	30,3	304,2±120,1 [b]	40,5	328,1±160,5 [b]	17,1
	S3	329,8±183,6 [b]	-4,3	385,7±226,3 [c]	-26,7	273,8±99,6 [c]	16,5
MMP-8	S1	245,9±122,4 [a]	---	276,1±136,8 [a]	---	215,7±97,0 [a]	---
	S2	228,9±75,7 [b]	6,9	238,6±38,2 [b]	13,5	219,1±99,1 [a]	-1,6
	S3	223,3±69,0 [b]	2,4	268,7±49,0 [a]	-12,6	177,8±54,8 [b]	18,8

Continua

Table 1 – PIC and MMps (pg/mL) concentrations, standard deviation and percentage reduction achieved by the use of different irrigants and intracanal medication during endodontic therapy of teeth with post-treatment apical periodontitis.

MMP-9	S1	129,4±29,6	a	---	124,1±35,1	a	---	134,7±21,5	a	---
	S2	121,6±45,6	b	6,0	84,3±43,5	b	32	131,9±45,4	a	2
	S3	122,9±93,1	b	-1	110,1±67,9	c	-30,6	135,7±111,3	a	-2,8
MMP-13	Initial	70,8±12,8	a	---	74,1±14,3	a	---	67,4±10,0	a	---
	CMP	60,9±8,7	b	13,9	64,8±8,5	b	12,5	57,0±7,1	b	15,4
	ICM	79,0±17,4	b	-29,7	85,9±11,4	c	-32,5	72,1±19,5	c	-26,5

PIC, proinflammatory cytokine; MMP, matrix metalloproteinase; CMP, chemomechanical preparation; ICM, intracanal medication. Different small letters indicate statistical differences at the intragroup level (column) considering $P < .05$.

Conclusão

Discussion

The major aim of the work was to quantify the proinflammatory cytokines (PIC) and matrix metalloproteinase (MMPs) present in the initial samples collected from the periapical region of teeth with endodontic failure. Moreover, it was also aimed to verify the effect of chemomechanical preparation (CMP) and intracanal medication (ICM) in change of the levels of PIC and MMPs. It was the first time that this methodology of collecting exsudate from the periapical fluids, via the root canals, was used for direct quantification of inflammatory contents. It is important to elucidate the expression profile of inflammatory molecules before endodontic treatment so that it is possible to understand the effect of intracanal procedures in molecular signals occurring in the apical periodontitis as well as the response from the host.

Proinflammatory cytokines levels during the endodontic therapy

TNF-α is an important PIC because it can modulate its expression as well as the other cytokines such as IL1-β (Tayal & Kalra 1999, Ataoglu et al. 2002, Hong et al. 2004, Martinho et al. 2011). It is also a major mediator of the inflammatory response to Gram-positive bacteria and other microorganisms (Safavi & Nichols 1994, Ataoglu et al. 2002, Hong et al. 2004). Its main biological function is the recruitment of neutrophils and monocytes to the site of infection and activation of these cells to eradicate the microorganisms (Martinho et al.,2012).

Regarding the role of IL1-β in the inflammatory process, it is capable of inducing the synthesis and release of mediators such as IL-6 and IL-8 and lead to the onset of hypotension, tachycardia, lactic acidosis, neutrophilia (Martinho et al. 2010). However, their most consistent biological effect is the increased synthesis of prostaglandin E_2 (PGE_2), which has the ability to sensitize nociceptors (Van Deuren et al. 1992). Particularly, IL-1β has been correlated with clinical signs/symptoms and greater bone resorption (Lim et al. 1994).

In our study, we observed in the initial samples (S1) lower levels of IL1-β (1.2 pg/mL) compared to TNF-α (8.8 pg/mL), confirming the regulatory role of the TNF-α in the inflammatory process.

In S2, CMP was able to significantly reduce PIC values (89.7% TNF-α and 91.6% IL1-β). However, after ICM there was an increase in all PIC levels. We believe that this increase is a maneuver of the organism to begin the tissue healing process, since the bacterial/ endotoxic content present in the root canals were reduced by the endodontic treatment.

Matrix metalloproteinase levels during the endodontic therapy

MMPs play an important role in the pathogenesis of pulp, periodontal and periapical tissue destruction (Tsai et al. 2005, Shin et al. 2011). PICs are able to stimulate direct and indirectly the release of MMPs in the periapical region, maintaining the persistent inflammatory process (Paula-Silva et al. 2010, Ozeki et al. 2015).

In the present study, different profiles of MMPs expression was observed in S1, being MMP-2 (collagenase; 803.7 pg/mL) and MMP-3 (stromelysin 1; 453.9 pg/mL) the most frequently found, followed by MMP-8 (245.9 pg/mL), MMP-9 (129.4 pg/mL) and MMP-13 (70.8 pg/mL). All of them participate in the degradation of the extracellular matrix (ECM) with different mechanisms of action and in different times of the inflammatory process.

In S2 (CMP) there was a slightly reduction of all MMPs levels, particularly of MMP-3 (30.3%). MMP-3 is an important enzyme in the degradation of the extracellular matrix, being able to degrade proteoglycans (i.e. fibronectin and laminin). On the other hand, it is also present in the epithelial cells during the process of tissue healing (Kusukawa et al. 1995, Ozeki et al. 2015), and in the osteoblast differentiation (Ozeki et al. 2014, Ozeki et al. 2015). Considering the hypothesis that this enzyme has regenerative effects, even though it has reached the highest percentual reduction during CMP, its residual values (329.8 pg/mL) could still act as a beneficial factor for tissue healing. However, there are no controlled clinical studies considering this enzyme as a positive factor for the bone remodeling process.

In S3 (ICM) there was a slightly increase of all MMPs levels, particularly MMP-2.

MMP-2 and MMP-9 are known as gelatinase. MMP-2 (gelatinase A or IV collagenase type/72 κDa) and MMP-9 (gelatinase B or IV collagenase type/92 κDa), degrade denatured collagen (gelatin), elastin, fibronectin, proteoglycans, laminin and

type IV collagen, which is the main component of the basement membrane, indicating involvement of these enzymes in the invasion process (Bauvois 2012). These gelatinases are particularly expressed in cases of granulomas and cysts, being also present in the gingival crevicular fluid of teeth with chronic periapical lesions (Carneiro et al. 2009, Dezerega et al. 2012).

In our study, CMP and ICM failed to reduce considerably the levels of MMP-2 and MMP-9, thus indicating that these molecules possess a broad pattern of expression in the pathological processes. Our results showed higher levels of MMP-2 than of MMP-9, agreing with the findings of Ahmed et al. (2013), who found that the MMP-9 expression is high in teeth with symptomatic apical periodontitis, stimulated by Gram-negative bacteria. The higher levels of MMP-2 found in teeth with asymptomatic post-treatment periodontitis may indicate that MMP-2 can play a role as a marker of chronic inflammatory process.

Regarding the MMP-8 and MMP-13, known as collagenase, they play a role particularly at the beginning of the inflammatory process. MMP-8 (neutrophil collagenase) and MMP-13 (collagenase 3) are enzymes responsible for the breakdown of type I and III collagens, which are major organic constituents of the alveolar bone and periodontal ligament (Hannas et al. 2007)

. The IL-1β present in inflammatory processes is a potent stimulator of MMP-8 and MMP-13, which are directly connected with the establishment and remodeling of periapical lesions (Matsui et al. 2011, Ozeki et al. 2014). Wahlgren et al. (2002) suggested that the levels of MMP-8 and MMP-13 from the periapical exudate could be used to indicate the status of the inflammatory response in order to predict a successful treatment of teeth with periapical lesions.

Shin et al. (2011) reported a correlation between MMP-8 and expression of substance P in symptomatic teeth. On the other hand, Geijersstam et al. (2005) suggested that in cases of endodontic failure, which involves a higher prevalence of Gram-positive bacteria and absence of symptomatology, there is a lower expression of MMP-8. Our results agree with the findings of Geijersstam et al. (2005), as the cases were asymptomatic, with a predominance of Gram-positive microorganisms.

In the present work, the overall levels of collagenases (MMP-8 and MMP-13) were lower than the gelatinases (MMP-2 and MMP-9), probably because the former enzymes are usually more active at the early stage of periapical lesion formation.

In general, this *in vivo* study was important to provide knowledge about the clinical levels of PIC and MMPs in the periapical region of teeth with post-treatment apical periodontitis, thus allowing further comparisons with different pathological situations involving the pulp and periapical tissues. In conclusion, regardless of the chemical substance tested, CMP is effective in reducing PIC and MMPs. The ICM increases the levels of TNF-α, IL-1β, MMP-2 e MMP-13.

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The authors deny any conflicts of interest related to this study.

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CAPÍTULO III

Antimicrobial susceptibility and characterization of virulence genes of *Enterococcus faecalis* isolates from teeth with failure of the endodontic treatment

Abstract

Aim: To investigate the prevalence of virulence factors and the antimicrobial resistance of *Enterococcus faecalis* isolates of teeth with failure of the endodontic treatment. **Methods**: Twenty root canal samples were collected from teeth with apical periodontitis. *E faecalis* was firstly presumpted identified based on phenotypic features and then by 16S rRNA gene sequencing. The antimicrobial susceptibility was determined by the minimum inhibitory concentration (MIC) of Amoxicillin (AC), Amoxicillin + clavulanate (XL), Azithromycin (AZ), Benzylpenicillin (PGL), Ciprofloxacin (CI), Clindamycin (CM), Chloramphenicol (CL), Doxycycline (DC), Erythromycin (EM), Gentamicin (GM), Metronidazole (MZ), Moxifloxacin (MX), Rifampicin (RI), Tetracycline (TC), Vancomycin (VA) using the E-test method. Virulence factors (*ace, asa, asa373, cylA, efaA, esp* and *gelE*) were detected by PCR assay. **Results**: XL was effective against all strains. Intermediate and total resistance was found against the majority of the tested antimicrobials. The susceptibility of some microorganisms to some antimicrobial agents changed according to the evaluation time. MIC_{50} and MIC_{90} also varied according to the evaluation time. In relation to the virulence factors of the *E. faecalis* isolates, *ace* was detected in 100% of the strains, *asa* (60%), *asa373* (15%), *esp* (70%) and *gelE* (75%); while *cylA* and *efaA* genes were not detected. **Conclusions**: It was concluded that *E. faecalis* isolates from persistent endodontic infections showed varied degrees of intermediate/ total resistance to several antimicrobial agents, being Amoxicillin + clavulanate the most effective agent. Moreover, the strains showed different patterns for virulence gene detection.

Keywords: Bacteria. Enterococcus faecalis. Microbial sensitivity tests. Virulence factor.

Introduction

Bacteria and their virulence factors are the main agents for the emergence of post-treatment apical periodontitis. They may have survived to the chemomechanical procedures or invaded the canal via coronal leakage of the root filling. Bacterial cultures and molecular studies have confirmed that *Enterococcus faecalis* is one of the most prevalent bacteria found in the root canal after endodontic treatment (1,2). *E. faecalis* are Gram-positive cocci and due to their morphological and genetic characteristics, can resist to intracanal procedures and systemic antibiotics, even in ecological conditions of stress (3).

The resistance mechanisms of *E. faecalis* result from physiological or structural changes in the bacterial cell, which is a survival strategy to abusive attack by antimicrobial agents (2). *Enterococcus* spp. has acquired genetic determinant conferring resistance to several classes of antibiotics, including Clindamycin, Erythromycin, Tetracycline, Chloramphenicol and, more recently, Vancomycin (2,4-6). Although the incidence of resistant strains is more pronounced in hospital or systemic infections, studies using bacterial isolates of endodontic infections have shown the emergence of bacterial resistance, especially at conventional regimen used in dental procedures (6).

Although systemic antibiotics are not commonly used in the treatment of intracanal infections associated with chronic periapical lesions, in cases of flare up, patients at risk of bacterial endocarditis development, these become an important adjunct to endodontic treatment, being used in prophylactic regimens (2). Systemic antibiotics act as an adjunct to the conventional surgical methods and should be used with restraint because of the possibilities of allergic reactions, toxicity, side effects and development of resistant strains of microbes (2,6). Therefore, it is imperative to monitor the *E. faecalis* resistance against the main antibiotics used in endodontics in order to provide updated data to guide physicians for the most effective therapy (6). Periodic and accurate antimicrobial susceptibility information is necessary to guide therapy and to call attention to the problem of antimicrobial resistance.

Epsilometer test (E test), an agar diffusion susceptibility test, holds the promise of being accurate and flexible enough to be performed in the most clinical laboratories (7), being used in several studies (1,6,8).

Virulence factors are measures that microorganisms have to facilitate adherence, colonization, resistance, pathogenicity and evasion of host immune response (3). The role of virulence factors of *Enterococcus* spp. it has not been fully elucidated and has attracted attention due to its ability to enhance the infection and to generate exacerbated responses. These strains, even in the presence of a restricted nutritional environment, may possess diversified mechanisms of virulence dependent on the genetic exchange process between them during the infection process (9). The virulence factors more often related to the *E. faecalies* are: *ace* (collagen binding protein), *asa* and *asa373* (aggregation substance), *cylA* (hemolysin activator), *efaA* (antigen endocarditis), *esp* (protein surface) and *gelE* (gelatinase). The expression of these genes in endodontic biofilm can enable or exacerbate distinct tissue responses in the periapical region, so it is imperative to understand the specific role of each in the pathogenicity of the infectious contents of the root canals.

Therefore, the purpose of this study was to analyze the antimicrobial susceptibility against antibiotics prescribed in endodontics by E-test and determine the prevalence the virulence factors of strains of *E. faecalis* isolates of post-treatment apical periodontitis.

Materials and Methods

Patient Selection

Twenty patients were selected from those who attended the Piracicaba Dental School, State University of Campinas- UNICAMP, Piracicaba, SP, Brazil, with a need for nonsurgical endodontic retreatment.

The Human Volunteers Research and Ethics Committee of the Piracicaba Dental School approved a protocol (# 018/2014), describing the specimen collection for this investigation, and all patients signed an informed consent for their participation in this research. The age of the patients ranged from 30 to 60 years. All selected teeth had been previously single root-filled and showed radiographic evidence of apical periodontitis.

Failure of root canal treatment was determined based on clinical and radiographic examinations. Presence of persistent periapical radiolucent lesion, voids in or around the root canal filling, persistent symptoms such as pain of palpation, discomfort to percussion, persistent sinus were considered reasons for retreatment (1).

Exclusion criteria were as follows. Subjects who had received antibiotic treatment within the preceding three months; reported systemic disease starting with ASA 3 (American Society of Anesthesiology); teeth that could not be isolated with rubber dam and teeth with periodontal pockets deeper than 3 mm were excluded.

Endodontic sample collection and clinical procedures

The teeth were isolated with rubber dam. The crown and surrounding structures were disinfected with 30% hydrogen peroxide (volume/volume for 30 seconds) followed by 2.5% sodium hypochlorite (NaOCl) for the same period of time and then inactivated with 5% sodium thiosulfate (10). Disinfection of the external surfaces of the crown was checked by taking a swab sample from the crown surface and streaking it onto blood agar plates, which were then incubated aerobically and anaerobically.

The sampling procedures were performed according to Martinho and Gomes (10). Under anesthesia (2% lidocaine with 1:100,000 epinephrine), a two-stage access preparation was performed. The access cavity was made without the use of water spray but under manual irrigation with sterile saline and by using sterile high-speed diamond bur. This first stage was performed to promote a major removal of contaminants. In the second stage, before entering the pulp chamber, the access cavity was disinfected according to the decontamination protocol described above. Disinfection of the internal surface of the access cavity was checked as previously described, and all procedures were performed aseptically. Root-filling materials were removed by using Reciproc R25 files (VDW, Munich, Germany) in the working lenght obtained by preoperatory radiography and used according to the manufacturer's instructions in a crown-down technique, with no chemical solvent.

Before sampling the root canal, a K-file #20 (Dentsply Maillefer, Ballaigues, Switzerland) was used to confirm the working length (previously estimated by radiographs), with an apex locator (Novapex; Forum Technologies, Rishon le-Zion, Israel). Three sterile paper points (Dentsply-Maillefer, Ballaigues, Switzerland) were

then consecutively introduced into the full length of the canal and retained in position during 60 seconds each. Next, the paper points were placed in a sterile tube containing 1 mL of VMGA III transport medium (11) for microbial culture. The transport medium samples were transported within 15 minutes to an anaerobic workstation (Don Whitley Scientific, Bradford, UK) for bacterial culture analysis.

Root canals were then prepared with 2% chlorhexidine gel or 6% NaOCl and by Reciproc R40 files (VDW, Munich, Germany) according to the manufacturer's instructions in a reciprocating working motion generated by the motor. These procedures were repeated until the Reciproc instrument reached the WL (zero point displayed on the apex locator).

Retreatment was deemed complete when the Reciproc file reached the working length, with no filling material covering the instrument and canal walls being smooth and free of visible debris. Furthermore, a close inspection under high magnification with dental operating microscope (DF Vasconcelos S/A, São Paulo, Brazil) showed complete removal of gutta-percha.

A final irrigation with 3 mL of 17% EDTA was applied continuously for 3 minutes under stirring with ultrasound (Advanced SE, Microdont, São Paulo, SP, Brazil) with tip ET40 (Satelec / Acteon, Mount Laurel, NJ) during 60 seconds alternately, followed by 5 mL of sterile saline. Finally, all teeth were dried and filled with single gutta-percha cone (Dentsply Maillefer, Ballaigues, Switzerland) and Endométhasone sealer (Septodont, Saint-Maur-des-Fossés, France). Access cavities were restored with Coltosol (Coltène/Whaledent) at a thickness of at least 2 mm and a second layer of composite material (Filtek Z250; 3M ESPE, St. Paul, MN, USA) was applied in combination with single bond adhesive.

Microbial culturing and molecular identification of E. faecalis Isolates by 16S rRNA gene sequencing

Microbial samples, isolation and speciation were done using advanced microbiologic techniques for anaerobic species.

Inside the anaerobic workstation, the tubes containing the transport medium were shaken in a mixer for 60 s. Serial 10-fold dilutions were made up to $1/10^4$ in pre-reduced Fastidious Anaerobe Broth (FAB, Laboratory M, Bury, UK) and 50 µL of each serial dilution were plated onto several media, as follows: 5% defibrinated

sheep blood-FAA Agar (FAA, Laboratory M, Bury, UK) alone, and supplemented with 600 µL of hemin and 600 µL of menadione. The plates were incubated at 37˚C in an anaerobic atmosphere for up to 48 hours to allow anaerobic or facultative microorganisms growth. In addition, 50 µL of initial sample was plated onto m-Enterococcus agar (Difco, Maryland, USA) and Mitis salivarius agar (Difco, Maryland, USA) to increase the chance of finding *E. faecalis*.

Preliminary characterization of microbial special was based on the features of the colonies (i.e. size, color, shape, high, lip, surface, texture, consistency, brightness and hemolysis), visualized under a stereoscopic lens (Lambda Let 2, Atto instruments Co., Hong Kong) at 16x magnification. Isolates were then purified by subculture, gram-stained, tested for catalase production, and their gaseous requirements established by incubation for 2 days aerobically and anaerobically. Based on this information it was possible to presumably identify *E. faecalis* (Gram-positive cocci, catalase-negative).

The DNA from the microorganisms presumably identified as *E. faecalis* was extracted and purified using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA concentration (absorbance at 260 nm) was determined using a spectrophotometer (Nanodrop 2000; Thermo Fisher Scientific, Wilmington, DE). After amplification of the 16S rRNA gene (forward primer: 5'GAG AGT TTG ATY MTG GCT CAG -3'; reverse primer: 5' - GAA GGA GGT GWT CCA RCC GCA – 3') (Invitrogen, São Paulo, São Paulo, Brasil) in the thermocycler (GenePro Thermal Cycler; Bioer Technology, Hangzhou, China), the purified product (QIAquick® Gel Extraction, Qiagen) at a concentration of 40ng/µL was subjected to sequencing by BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, California, USA), following the manufacturer's recommendations. The sequences obtained were first analyzed for quality and similarity of nucleotides, using CLC Genomics Workbench software v6.5 (Qiagen). Those considered of good quality were aligned using the 6 Mega software (Molecular Evolutionary Genetics Analysis) and thus, compared with the sequences deposited in GenBank, the Basic Local Alignment Tool program for Nucleotide (BLASTn). For a high level of quality of the sequences, a confidence level of 99-100% similarity to *E. faecalis* was established (12).

Antimicrobial Susceptibility Test

The susceptibility of *E. faecalis isolates* was determined by the minimum inhibitory concentration (MIC) of different antibiotics by using the E-test method (BioMérieux SA, Marcy-l'Etoile, France).

Colonies were suspended in Brucella broth to achieve a density corresponding to 1.0 McFarland turbidity standard. A cotton wool swab soaked in the inoculum was used to inoculate the surface of plates containing 5% defibrinated sheep blood Brucella agar enriched with 5 mg/mL of hemin and 1 mg/mL of vitamin K. The E-test strip was applied separately to the center of the plate with the high-MIC end toward the edge of the plate. The plates were then immediately incubated in an anaerobic chamber (Don Whitley Scientific, Bradford, England) for 24–48 hours. After growth, an ellipse of inhibition was seen around the strip. At the point of intersection of the ellipse with the strip, the MIC was read from an interpretative scale (BioMérieux SA) (Figure 1).

MIC less than or equal to the breakpoints recommended by the Clinical Laboratory Standard Institute (CLSI, 2013) were considered susceptible; those above the breakpoints were considered resistant. The reference values are shown in Table 1. The MICs of the antibiotics that inhibited 50% and 90% of the isolates were calculated and expressed as MIC_{50} and MIC_{90}, respectively.

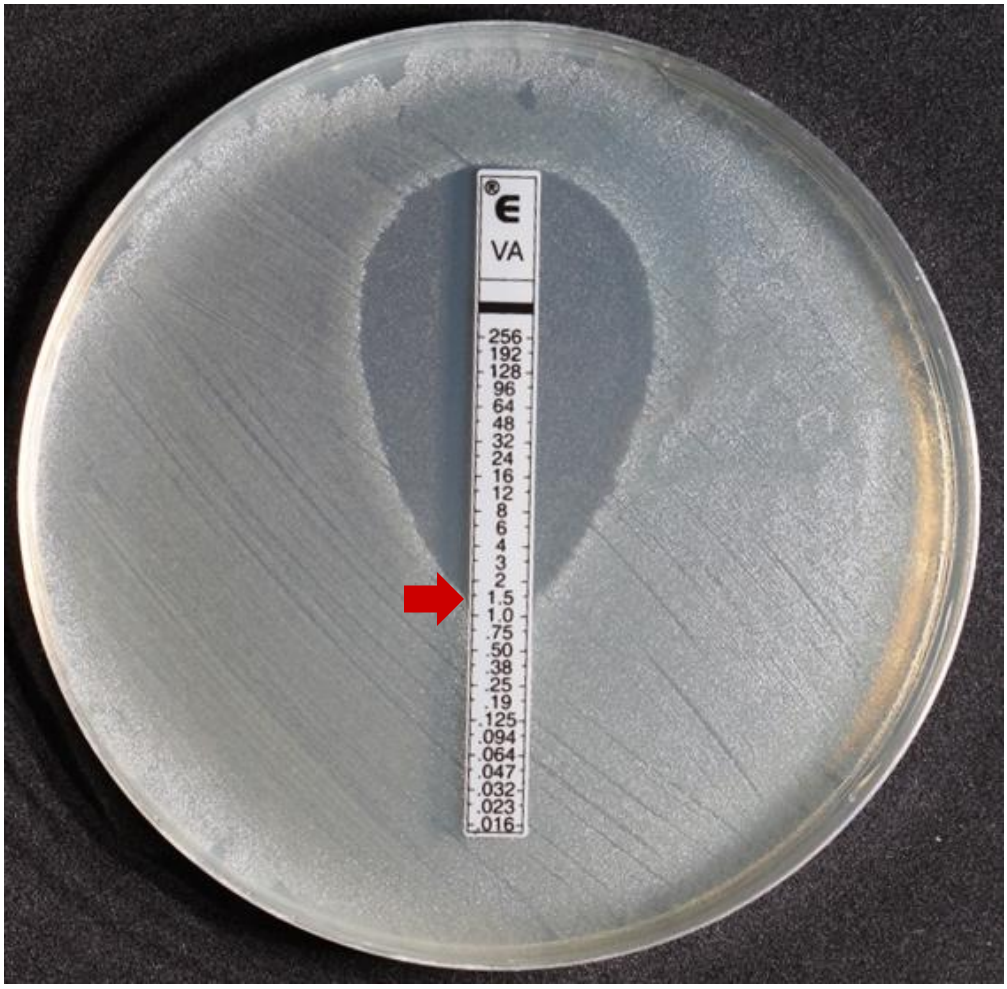

Figure 1. E-test method. Plate with bacterial growth of *Enterococcus faecalis* and vancomycin strip after 48-hour incubation. The arrow shows the intersection point between the

Table 1 – Range of concentration of antibiotics in the E-test strip and interpretative values cutoffs equivalent of the minimum inhibitory concentration (MIC) (µg/mL) of antimicrobial evaluated in *Enterococcus faecalis* tests (CLSI, Clinical Laboratory Standard Institute, 2013).

Antimicrobial agents	Range (µg/mL)	Susceptible	Intermediate	Resistant
Amoxicillin	(256 – 0.016)	≤2	4	≥8
Amoxicillin + clavulanate	(256 – 0.016)	≤4	8	≥16
Azithromycin	(256 – 0.016)	≤2	4	≥8
Benzylpenicillin	(256 – 0.016)	≤8	12	≥16
Ciprofloxacin	(32 – 0.002)	≤1	2	≥4
Clindamycin	(256 – 0.016)	≤2	4	≥8
Chloramphenicol	(256 – 0.016)	≤8	16	≥32
Doxycycline	(256 – 0.016)	≤4	8	≥16
Erythromycin	(256 – 0.016)	≤0,5	1-4	≥8
Gentamicin	(256 – 0.016)	≤4	8	≥16
Metronidazole	(256 – 0.016)	≤8	16	≥32
Moxifloxacin	(32 – 0.002)	≤2	4	≥8
Rifampicin	(32 – 0.002)	≤1	2	≥4
Tetracycline	(256 – 0.016)	≤4	8	≥16
Vancomycin	(256 – 0.016)	≤4	8-16	≥32

PCR assay for virulence gene detection

PCR reactions for the detection of virulence genes of *E. faecalis* were performed in a thermocycler (GenePro Thermal Cycler) according to Sedgley et al. (13). It was used a total volume of 30 µL containing 3 µl 10X PCR buffer (Invitrogen), 100 µM dNTPs mix (Invitrogen), 2,4 µL 25 mmol/L $MgCl_2$, 6 pmol of each respective primer (Invitrogen), 100–200ng total DNA template, 2 U Platinum Taq DNA polymerase (Invitrogen), and nuclease-free water. The PCR conditions were as follows: 15 min initial enzyme activation/DNA denaturation step at 95°C followed by 35 consecutive cycles at 94°C for 20 s; 58°C for 45 s; 72°C for 60 s. The primer sequences are listed in Table 2.

PCR products were analyzed by electrophoresis using 1% agarose gels (containing ethidium bromide) in TBE buffer. Gels were analyzed under ultraviolet (UV) light. The size of fragments generated by PCR was compared with the 1 Kb-Plus ladder (Invitrogen). The detection of target-genes was considered when there was the presence of positive bands (Figure 2).

Table 2. Pair of oligonucleotides (forward and reverse) used in the polymerase chain reaction (PCR) for detection of virulence genes of *E. faecalis* isolated from teeth with post-treatment apical periodontitis.

Gene	Primers pairs (5'- 3')	Amplicon size (bp)
ace (collagen-binding protein)	F: GGAATGACCGAGAACGATGGC R: GCTTGATGTTGGCCTGCTTCCG	616
asa (aggregation substances)	F: CCAGCCAACTATGGCGGAATC R: CCTGTCGCAAGATCGACTGTA	529
asa373 (aggregation substances)	F: GGACGCACGTACACAAAGCTAC R: CTGGGTGTGATTCCGCTGTTA	619
cylA (haemolysin activator)	F: GACTCGGGGATTGATAGGC R: GCTGCTAAAGCTGCGCTTAC	688
efaA (endocarditis antigen)	F: GCCAATTGGGACAGACCCTC R: CGCCTTCTGTTCCTTCTTTGGC	688
esp (surface protein)	F: TTGCTAATGCTAGTCCACGACC R: GCGTCAACACTTGCATTGCCGA	932
gelE (gelatinase)	F: ACCCCGTATCATTGGTTT R: ACGCATTGCTTTTCCATC	405

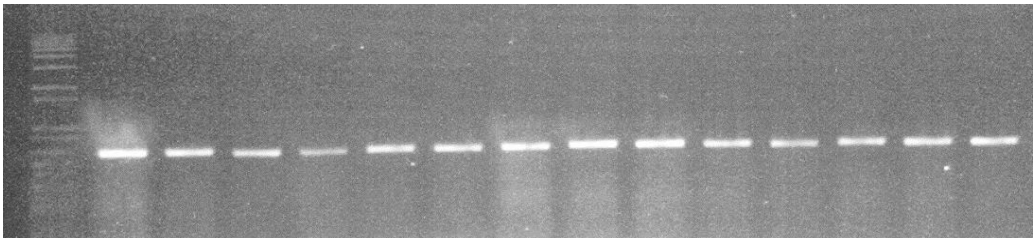

Figure 2. Capture of the photograph of the 1% agarose gel, showing the positive bands for virulence gene tested by PCR assay.

Results

E. faecalis was present in 100% of the root canals investigated. One strain per canal was selected for this study, totalizing 20 isolates.

Different susceptibility patterns were found for the *E. faecalis* isolates. All strains were susceptible (S) to Amoxicillin + clavulanate. Some strains showed intermediary (I) susceptibility to Amoxicillin (5%), Azithromycin (20%), Benzylpenicillin (5%), Ciprofloxacin (15%), Doxycycline (5%), Erythromycin (75%), Tetracycline (10%) and Vancomycin (15%). The other antibiotics had less favorable results, exhibiting resistance, such as Clindamycin (60%), Chloramphenicol (5%), Gentamicin (65%), Metronidazole (95%), Moxifloxacin (5%) and Rifampicin (10%). to some antimicrobial agents. The MIC_{50} and MIC_{90} values are listed in Table 3.

In relation to the virulence factors of the *E. faecalis* isolates, *ace* was detected in 100% of the strains, *asa* (60%), *asa373* (15%), *esp* (70%) and *gelE* (75%); while *cylA* and *efaA* genes were not detected.The results found for virulence genes are listed in Table 4.

Table 3. Antimicrobial activities of various antimicrobial agents (µg/mL) against *Enterococcus faecalis* isolates (n=20) of teeth with endodontic treatment failure.

Antimicrobial agents	**MIC**			**Susceptibility rate (%)**		
	Range	MIC_{50}	MIC_{90}	S	I	R
Amoxicillin	0.13 - 5.33	0.38	0.5	95%	5%	-
Amoxicillin + clavulanate	0.09 - 2.67	0.4	0.88	100%	-	-
Azithromycin	0.48 - 14.67	2.67	13.3	80%	20%	-
Benzylpenicillin	0 - 9.33	0.92	2.17	95%	5%	-
Ciprofloxacin	0.007 - 2	0.63	1.83	85%	15%	-
Clindamycin	0.01 - 64	29.3	64	30%	10%	60%
Chloramphenicol	0.19 - 14.67	4	7.33	95%	-	5%
Doxycycline	0.02 - 6	0.25	0.71	95%	5%	-
Erythromycin	0.02 - 6.7	0.92	3	25%	75%	-
Gentamicin	0.02 - 18.67	4	18.67	25%	10%	65%
Metronidazole	0.05 - 1.83	1.83	1.83	5%	-	95%
Moxifloxacin	0.04 - 12	0.21	0.42	95%	-	5%
Rifampicin	0.01 – 3.3	1.5	3.33	25%	65%	10%
Tetracycline	0.03 - 14.67	0.63	1	90%	10%	-
Vancomycin	1.25 - 6	2.33	5.33	85%	15%	-

MIC, minimum inhibitory concentration; MIC_{50}, MIC for 50% of the strains; MIC_{90}, MIC for 90% of the strains; S, susceptible; I, Intermediate; R, resistant.

Table 4. Prevalence of virulence genes of *Enterococcus faecalis* isolates teeth with post- apical periodontitis detected by PCR assay.

Strain	**Gene**				
	ace	asa	asa373	esp	gelE
Isolate 1	+	-	-	+	-
Isolate 2	+	-	-	+	-
Isolate 3	+	-	-	+	-
Isolate 4	+	-	-	+	-
Isolate 5	+	+	-	+	+
Isolate 6	+	-	-	+	+
Isolate 7	+	+	-	+	+
Isolate 8	+	+	-	+	+
Isolate 9	+	+	-	+	+
Isolate 10	+	+	-	+	+
Isolate 11	+	-	-	+	+
Isolate 12	+	-	-	-	+
Isolate 13	+	+	+	+	+
Isolate 14	+	+	+	-	+
Isolate 15	+	+	-	-	+
Isolate 16	+	+	-	+	-
Isolate 17	+	+	+	-	+
Isolate 18	+	+	-	-	+
Isolate 19	+	+	-	+	+
Isolate 20	+	-	-	-	+

Discussion

Antimicrobial Susceptibility

E. faecalis is a Gram-positive bacterium that has different mechanisms of virulence and resistance that make it difficult to be eradicated from the root canals. One of the objectives of this study was to evaluate the susceptibility of *E. faecalis* strains against several antimicrobial agents in order to monitor their susceptibility/resistance over time. Gomes et al. (6), monitoring the primary endodontic infections found that some bacteria showed an increase in resistance against the antibiotics tested over time. Our study, involving *E. faecalis* isolated from root canals of secondary/persistent endodontic infections also showed different profiles of susceptibility.

The E-test, recommended by the Clinical and Laboratory Standards Institute (CLSI), is a standard method broadly used in medical microbiology, easy to use and interpret, in addition to being safe for testing antimicrobial susceptibility of bacteria involved in endodontic infections (6,8,14-16). The CLSI periodically revises and determines the standards for both testing and interpretation of the results, allowing the determination of the MIC for the tested strains. The method can also provide data for comparison in longitudinal studies assessing changes in the bacterial susceptibility profiles over time (6,8).

In the present work amoxicillin + clavulanate was effective against all tested isolates; followed by amoxicillin and benzylpenicillin which were effective against the majority of the strains, corroborating with other studies in the literature (1,17-18). However, the presence of enterococcal strains resistant to penicillin has been reported in endodontic infections (19) which underlines the need to perform periodical susceptibility tests of these isolates.

Due to the predominance of *E. faecalis* in root-filled teeth with periapical lesions, alternative drugs should be considered for prophylaxis in individuals at risk for endocarditis during endodontic retreatment or with flare up (20). In cases of patients allergic to penicillin and its derivatives, clindamycin is the antibiotic most commonly prescribed. However, clindamycin is not an alternative drug to treat *E faecalis* infections in patients allergic to penicillin and its derivatives. According to

CLSI, aminoglycosides (e. g. gentamicin) (except in high concentrations), cephalosporins, clindamycin, and trimethoprim-sulfametroxazol may appear active in vitro but are not clinically effective and should not be reported as sensitive. We found that gentamicin and clindamycin are not effective against *E. faecalis* agreeing with the findings of several authors (5, 20).

We found a high rate of resistance to clindamycin, gentamicin, metronidazole and more low the rifampicin, agreeing with Zhu et al. (21) and Sun et al. (22), but disagreeing with other studies in the literature (13,23). This fact may suggest the development of resistance of these bacteria over the years (Gomes et al., 2011).

Regarding azithromycin and erythromycin we found an percentual of intermediary resistance of 25% and 75%, respectively, agreeing with the findings of Endo et al. (2), who found very similar values (33.3% and 75%, respectively). Pinheiro et al. (20) reported that 14.2% and 28.5% of *E. faecalis* strains were susceptible to azithromycin and erythromycin, respectively. Nevertheless, in the present study none of the *E. faecalis* strains studied demonstraded to be susceptible to both antibiotics. Emerging antibiotic resistance in *Enterococcus* spp. has been shown in recent studies (24). Enterococci have acquired genetic determinants that confer instrinsic resistance to many classes of antimicrobials, including tetracycline, erithomycin and chloramphenicol (4).

The chloramphenicol showed a good antimicrobial activity against *E. faecalis* (95%), agreeing with the findings of other studies (25-26), demonstrating that this antibiotic can be an alternative drug in cases where the derivatives of penicillin failed.

The doxycycline, moxifloxacin tetracycline and vancomycin also demonstrated a good action against *E. faecalis*, which may suggest the use of these drugs as an alternative from therapy. However, their use should be undertaken with caution in order to avoid bacterial resistance development (20,26).

As an alternative therapy for patients allergic to penicillin, some authors (1,2) suggested the use of moxifloxacin, because they found 100% susceptibility to this antibiotic to *E. faecalis*. However, our study has shown that there is already a change in the pattern of susceptibility over time, because the susceptibility of rate has already dropped to 95% and one strain showed total resistance to moxifloxacin. Meanwhile, our study indicates the use of doxycycline for patients allergic to penicillin, because this antibiotic showed antimicrobial activity for 95% of the strains and only one strain showed intermediary resistance.

In relation to the antibiotic therapy, an endodontic infection must be persistent or systemic to justify the need for antibiotics, i. e. fever, swelling, lymphadenopathy, trismus or malaise in a healthy patient (27). Antibiotics are also more likely to be needed in an immunocompromised (28) patient or a patient in poor health.

The increasing resistance to antimicrobial agents is a concern as it can have a negative effect on treatment effectiveness, increasing the risk of superinfections.

E. faecalis virulence factors

This study found distinct prevalence patterns of the virulence genes of *Enterococcus* spp isolated from root canals of teeth with failure of the endodontic treatment.

The *ace* gene was detected in all clinical isolates. *Ace* is an adhesin to collagen from *E. faecalis* expressed conditionally after growth in serum or in the presence of collagen. It is an important factor for the establishment of these bacteria in the dentin of infected root canals (29).

GelE gene was detected in 75% of the *E. faecalis* strains, agreing with the findings of Zhu et al. (21). Gelatinase (*gelE*) is a hydrophobic metalloprotease with the capacity for cleaving insulin, casein, hemoglobin, collagen, gelatin and fibrin. The expression of *gelE* was higher in the biofilm-positive than in biofilm-negative strains (9,30)

The *asa* (60%) and *esp* (70%) genes also had high prevalences in our study, corroborating other studies in the literature (13,29).

Aggregation substance (*asa*) is a pheromone-responsive, plasmid- encoded bacterial adhesin that mediates efficient contact between donor and recipient bacterium, facilitating plasmid exchange. *Asa* was also found to mediate binding to extracellular matrix proteins, including collagen type I. Binding to collagen type I by bacteria may be of particular importance with respect to endodontic infections, since this is the main organic component of the dentin (21,31).

Asa373 was identified in 15% of the isolates disagreeing with the findings of Sedgley et al. (13) who had not detected this gene. *Asa373*, differs in its protein structure from the classic *asa* and was reported to exhibit some moderately conserved amino acid motifs, when its database sequence was compared with those of some other bacterial adhesins (32).

The enterococci surface protein (*esp*) is encoded by the *esp* gene and may be involved in colonization and persistence of *E. faecalis* during infections (33). Enterococcal gene *esp*, encoding the high-molecular-weight surface protein *esp*, has been detected in abundance among bacteremia and endocarditis isolates, but is rare in stool isolates from healthy individuals (34). It is likely that it mediates the primary interaction of the pathogen with host surfaces during biofilm formation (30).

Finally, the Cytolysin (*cylA*) and *efaA* genes were not detected, which is in disagreement with Sedgley et al. (13), who identified these genes in 18% and 100% of the cases, respectively. *CylA* can induce tissue damage through the lysis of erythrocytes and destruction of host cells. Sedgley et al. (13) determined 36% of the *E. faecalis* endodontitis-associated strains to be capable of producing hemolysin. The genes in the cyl operon encode cytolysin, where *cylA* is the only reading frame necessary for the expression of component A, a serine protease.

The endocarditis antigen (efaA) *gene* was previously identified with the use of an antiserum from a patient with *E. faecalis* endocarditis (35). The amino acid sequence of the associated protein, *efaA*, revealed 55 to 60% homology to a group of streptococcal proteins known as adhesins. Thus, it was hypothesized that e*faA* might be functioning as an adhesin in endocarditis. Production of *efaA* by strains of *E. faecalis* is common

The different expression profile of these virulence factors can be explained by geographical differences, dietary habits and infection stage (9,13). This can be important for understanding the pathogenicity of virulence factors and their effects on the host.

It was concluded that *E. faecalis* isolates from persistent endodontic infections showed varied degrees of intermediate/total resistance to several antimicrobial agents, being amoxicillin + clavulanate the most effective agent. The periodic evaluation of the susceptibility to antibiotics is an important practice for establish the best drug if their use is necessary. Moreover, the strains showed different patterns for virulence gene detection against *E. faecalis*. Your monitoring is encouraged in order to elucidate changes us their resistance profiles.

Acknowledgments

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The authors deny any conflicts of interest related to this study

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DISCUSSÃO

A particularidade da microbiota encontrada em canais radiculares com periodontite apical secundária é a resistência específica de microrganismos aos procedimentos endodônticos, incluindo sua capacidade de sobreviver a condições com recursos nutricionais limitados (Sundqvist et al., 1998; Nóbrega et al., 2013). Gomes et al. (2008) mencionaram que dentes com insucesso do tratamento endodôntico tem 9 vezes mais chance de apresentar *Enterococcus faecalis* em relação aos casos de infecção primária. Devido as características de resistência deste microrganismo muitos autores consideram o *E. faecalis* o principal patógeno envolvido na periodontite apical pós-tratamento endodôntico (Pinheiro et al., 2003ab; Gomes et al., 2008; Zhang et al, 2012; Endo et al., 2012, 2013; Rahimi et al, 2014; Tay et al, 2015).

Os mecanismos de colonização, resistência e virulência dos microorganismos determinam o padrão de infecção endodôntica através da ativação de uma rede de mediadores que são capazes de instalar e perpetuar a um processo inflamatório na região periapical. O papel dos microrganismos e de seus sub-produtos em infecções endodônticas primárias são bem elucidados na literatura científica. No entanto, em casos onde houve falha do tratamento endodôntico, há uma escassez de estudos que investigaram o efeito dos procedimentos intracanais no controle deste conteúdo infeccioso (Baugh e Wallace, 2014; Gade et al., 2013; Miranda et al., 2013; Koçak et al., 2014; KungWani et al., 2014).

Através dos seus fatores de virulência, *E. faecalis* cria uma série de injúrias ao tecido do hospedeiro, incluindo a destruição da matriz extracelular através da produção de metaloproteinases de matriz. Estas moléculas são expressadas particularmente pela indução das citocinas pró-inflamatórias de TNF-α e lL1-β (Ryu et al., 2009; Costa, Souza-Filho e Barbosa, 2003; Ozeki et al., 2015).

O LTA é um factor de virulência importante das bactérias Gram-positivas. Por meio de diferentes mecanismos imunológicos, esta molécula pode ativar os mediadores químicos relacionados com a destruição óssea e sintomatologia dolorosa (Baik et al., 2008; Ryu et al., 2009). Portanto, é importante o monitoramento de seus níveis, a fim de controlar seus efeitos deletérios sobre os tecidos periapicais. Isso porque a presença aumentada de mediadores químicos, tais como citocinas

pró-inflamatórias, prostaglandinas e de espécies reativas de oxigénio, pode atuar como um fator determinante para o desenvolvimento ou perpetuação da periodontite apical pós-tratamento endodôntico (Costa, Souza-Filho & Barbosa, 2003; Zhao et al., 2013). A determinação dos níveis de LTA em dentes com insucesso endodôntico obtidos nesta pesquisa *in vivo* permitirá comparações futuras com outras patologias pulpares e periapicais. Poderá servir também de base para avaliar o efeito do tratamento endodôntico na diminuição da sintomatologia dolorosa e no favorecimento do reparo tecidual.

Em relação aos fatores de virulência testados foi possível observar a notável prevalência dos genes *ace, asa, esp* e *gelE*. Os distintos padrões de detecção revelam a capacidade mutacional dos *Enterococcus faecalis*. A expressão destes genes pelo biofilme bacteriano pode ocasionar a permanência da bactéria no interior do canal radicular (Reynaud af Geijersstam et al., 2007), como também sua capacidade em estimular destruição óssea na região periapical (Wang et al., 2011). Os diferentes perfis de expressão nas cepas clinicas isoladas pode ser reflexo das diferenças geográficas e hábitos alimentares dos pacientes (Sedgley et al., 2005; Wang et al., 2011). O constante monitoramento da expressão dos genes de virulência é importante para o entendimento da patogenicidade dos *Enterococcus* spp.

No presente estudo, os dentes com periodontite apical não apresentavam um quadro inflamatório agudo, por isso os níveis das citocinas testadas provavelmente são mais baixos comparados àqueles encontrados em processos agudos (Martinho et al., 2010, 2011, 2012). Ademais, não existem estudos na literatura quantificando citocinas em dentes com infecção persistente que permita a comparação desses parâmetros. Nesta pesquisa, foi realizada a quantificação direta das citocinas TNF-α e IL-1β através do ensaio imunoenzimático, permitindo desta forma mensurar os seus níveis e avaliar o efeito do preparo PQM e MIC na sua redução. Conhecer o papel das citocinas pró-inflamatórias na modulação da resposta imune nas patologias pulpares e perirradiculares pode contribuir para o entendimento da imunobiologia da periodontite apical e suas manifestações clínicas/radiográficas.

Alguns autores sugerem que as MMPs estão envolvidas na patogênese da periodontite apical (Paula-Silva, da Silva e Kapila, 2010; Matsui et al., 2011; Menezes-Silva et al., 2012) e que altos níveis dessas moléculas são relacionados a falta de cicatrização do tecido (Paula-Silva, da Silva e Kapila, 2010). No presente

estudo foi possível observar diferentes padrões de expressão de MMP, sendo a MMP-2 a que apresentou concentrações mais elevadas. MMP-2 (gelatinase A ou IV colagenase tipo / 72 κDa) e MMP-9 (gelatinase B ou tipo IV colagenase / 92 κDa) degradam o colagénio desnaturado (gelatina), elastina, fibronectina, proteoglicanos, laminina e colagénio tipo IV, que é o componente principal da membrana basal, indicando o envolvimento destas enzimas no processo de invasão (Bauvois, 2012). A literatura mostra que estas gelatinases são particularmente expressadas em casos de granulomas e cistos, estando também presentes no fluido gengival de dentes com lesões periapicais crônicas (Carneiro et al., 2008; Dezerega et al., 2012). MMP-8 (neutrofil coleganase) e MMP-13 (colagenase 3) são enzimas responsáveis pela quebra do colágeno tipo I e III, que são os principais constituintes orgânicos do osso alveolar e ligamento periodontal. Verificou-se também níveis elevados de MMP-3 (estromelisina 1), que é também uma enzima importante na degradação da matriz extracelular, mas que pode estar presente nas células epiteliais durante o processo de cicatrização do tecido, nestes casos atuando de forma benéfica aos tecidos (Kusukawa et al., 1995; Ozeki et al., 2015).

As técnicas atuais de instrumentação possibilitam um PQM mais rápido e com isso a substância química permanece menos tempo no interior do canal radicular. A presente pesquisa buscou utilizar uma concentração mais elevada do hipoclorito de sódio (6%) com o intuito de investigar se haveria uma maior redução do conteúdo infeccioso em comparação com a clorexidina gel 2%. Contudo, ambas as substâncias testadas foram capazes de reduzir estes níveis consideravelmente, visto existir uma combinação da ação química da substância química com a ação mecânica da irrigação e do instrumento endodôntico.

É importante salientar que, o sistema reciprocante de lima única “Reciproc” mostrou-se eficaz na redução do conteúdo infeccioso, corroborando com estudos prévios (Basmaci et al., 2013; Marinho et al. 2015. Martinho et al., 2014).

De uma forma geral, o PQM foi capaz de reduzir os níveis do conteúdo infeccioso/inflamatório, independente da substância utilizada, corroborando com os achados da literatura (Martinho et al., 2010; Marinho et al., 2014; Gomes et al., 2013; Martinho et al., 2014). Esta pesquisa também se destinou a avaliar o efeito complementar da MIC à base de $Ca(OH)_2$ para reduzir os níveis do conteúdo infeccioso/inflamatório dos canais radiculares com insucesso do tratamento endodôntico. A MIC apresentou padrões distintos de redução, tendo sido mais

significativa na redução de LTA e MMP-3/-8 (especificamente no grupo do NaOCl 6%). De uma forma geral houve aumento dos níveis de citocinas pró-inflamatórias e MMPs após a utilização da MIC, possivelmente pelo tempo prolongado de uso. Em contrapartida, a hipótese de que este aumento dos mediadores químicos após a remoção da maior parte do conteúdo infecioso dos canais radiculares sirva como suporte ao reparo tecidual, pode justificar os resultados encontrados neste estudo. Como não há estudos similares para comparação destes níveis, que são baixos comparados àqueles existentes em infecções primárias, parece cauteloso considerar que são aceitáveis, necessitando de mais pesquisas para a confirmação desta hipótese.

Os resultados desta pesquisa são esclarecedores do ponto de vista clínico e por ser inovador, impulsionam novos questionamentos e descobertas. Um dente que foi tratado endodonticamente e falhou requer um planejamento e execução de um novo tratamento que leve em consideração os fatores anatômicos, técnicos, microbiológicos e aqueles relacionados à resposta do hospedeiro, de forma a ser realizado dentro de padrões de alta qualidade e com recursos tecnológicos eficientes. Para tanto, é necessária a habilidade do operador, seleção correta do caso, técnica adequada e material a ser utilizado, para que seja possível ultrapassar o nível de qualidade do tratamento anterior; estabelecer a saúde perirradicular e apresentar meios que mantenham o dente protegido e em função no sistema estomatognático; e assim, conseguir alcançar o sucesso clínico, radiográfico e histológico, a curto, médio e longo prazo.

O monitoramento *in vivo* dos níveis bacterianos, de citocinas pró-inflamatórias, de LTA e de MMPs ao longo da terapia endodôntica foi importante para estabelecer parâmetros clínicos que permitam comparações futuras com diferentes patologias de origem endodôntica, e assim, melhor compreender as causas do insucesso endodôntico e quais os eventos inflamatórios envolvidos neste processo. Este controle é essencial para a compreensão dos efeitos deletérios que os microrganismos e seus fatores de virulência causam no estabelecimento da periodontite apical pós-tratamento endodôntico, através de diferentes eventos imunobiológicos.

CONCLUSÃO

Capítulo I

- 71,4% (70/102) das bactérias isoladas do canal radicular de dentes com insucesso endodôntico é Gram-positiva. Maior diversidade e número está presente nas amostras iniciais e *Enterococcus faecalis* é a bactéria mais prevalente (12/70 em C1, 3/70 em C2, e 70/70 em C3).

- O PQM foi eficiente em reduzir bactérias cultiváveis e LTA independentemente da substância química utilizada.

- A MIC foi efetiva para reduzir adicionalmente os níveis bacterianos somente no grupo do NaOCl 6%, pois o grupo da clorexidina 2% gel conseguiu resultados satisfatórios logo após o PQM.

- As taxas de redução de bactérias cultiváveis foram maiores em comparação com LTA e a fase com níveis mais consideráveis de redução do conteúdo infeccioso ocorreu logo após o PQM.

Capítulo II

- O PQM conseguiu reduzir os níveis de citocinas pró-inflamatórias (TNF-α e lL1-β) e de MMPs (-2, -3, -8, -9 e -13), enquanto que a MIC não foi efetiva em reduzir este conteúdo inflamatório.

- Após a utilização da MIC houve aumento dos níveis globais de TNF-α, lL1-β, MMP-2.

- Clorexidina 2% gel e NaOCl 6% foram efetivos na redução de TNF-α e lL1-β.

- A clorexidina 2% gel reduziu os níveis de todas as MMPs, ao passo que o NaOCl 6% foi efetivo somente na redução da MMP-2, MMP-3 e MMP-9.

- A MIC foi efetiva na redução de MMP-3 somente no grupo em que foi utilizado o NaOCl 6%.

Capítulo III

- Amoxicilina + ácido clavulânico apresentou atividade antimicrobiana sobre todas as cepas clínicas testadas.

- Amoxicilina, Azitromicina, Benzilpenicilina, Ciprofloxacina, Doxiciclina, Tetraciclina e Vancomicina apresentram atividade antimicrobiana efetiva sobre a maioria das cepas, enquanto que para outras apresentou ação intermediária.

- *Enterococcus faecalis* apresentou padrões variados de suscetibilidade e resistência a Clorafenicol, Erittromicina, Moxifloxacina e Rifampicina.

- Clindamicina, Gentamicina e Metronidazol obtiveram os piores resultados sobre *Enterococcus faecalis*, com o desenvolvimento de cepas resistentes.

- Apesar da pouca variação, houve mudança no padrão de atividade dos antibióticos entre os períodos testados (24 e 48 horas), variando entre suscetível, intermediário e resistente.

- O gene de virulência mais prevalente foi o *ace*, seguido de *asa*, *asa373*, *esp* e *gelE*. Enquanto que cylA e efaA não foram detectados.

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APÊNDICE 1

Aspectos clínicos e radiográficos de pacientes com insucesso do tratamento endodôntico.

Caso	Dente	Dor Prévia	Dor Atual	DP	SP	Edema	Fístula	TT (meses)	CO	TL	SC
1	35	+	-	-	-	-	-	180	-	10 x 8	+
2	45	-	-	-	-	-	-	420	-	5 x 2,5	+
3	21	+	-	-	-	-	-	216	-	3 x 3	+
4	34	+	-	-	+	-	-	60	-	3 x 3	+
5	21	-	-	-	+	-	-	396	-	3 x 3	+
6	45	-	-	-	+	-	-	120	-	3 x 3	+
7	35	-	-	-	+	-	-	120	-	4 x 4	+
8	25	+	-	-	-	-	-	144	-	4 x 3	+
9	35	+	-	+	+	-	-	120	-	6 x 3	+
10	22	-	-	-	+	-	-	72	-	2 x 2	+
11	21	+	-	+	+	-	-	24	-	2 x 1,5	+
12	21	-	-	-	-	-	-	84	-	1 x 1	+
13	15	-	-	-	+	-	-	180	-	3 x 2	+
14	45	-	-	-	-	-	-	60	-	3 x 5	+

Continua

Conclusão

Caso	Dente	Dor Prévia	Dor Atual	DP	SP	Edema	Fístula	TT (meses)	CO	TL	SC
15	22	-	-	+	-	-	-	40	-	3 x 3	+
16	23	-	-	+	-	-	-	40	-	1 x 1	+
17	11	-	-	-	+	-	-	108	-	3 x 3	+
18	21	-	-	-	+	-	-	100	-	3 x 3	+
19	22	-	-	-	+	-	-	115	-	4 x 2	+
20	41	-	-	-	+	-	-	180	-	6 x 5	+

DP, dor à palpação; SP, sensibilidade à percussão; TT, tempo de tratamento; CO, cortical óssea, TL, tamanho da lesão; SC, selamento coronário.

APÊNDICE 2

Bactérias Gram-positivas encontradas nas diferentes fases do retratamento endodôntico pelo teste bioquímico.

Fase		Microrganismo	G1	G2
Sample	S1	*Aerococcus spp*	7	5
		Enterococcus faecalis	8	4
		Staphylococcus spp	1	3
		Actinomyces naeslundii	1	2
		Bifidobacterium spp	3	0
		Eggerthella lenta	1	2
		Enterococcus spp	2	1
		Gemella spp	1	2
		Actinomyces israelii	1	0
		Actinomyces viscosus	1	0
		Clostidium histolyticum	0	1
		Gemella morbillorum	1	0
		Lactococcus lactis spp	0	1
		Micrococcus spp	0	1
		Staphylococcus capitis	1	0
		Staphylococcus xylosus	0	1
		Staphylococcus warneri	0	1
		Streptococcus salivarius	0	1
		Streptococcus uberis	0	1
	S2	*Enterococcus faecalis*	0	3
		Micrococcus spp	0	1
	S3	*Bifidobacterium spp*	0	1
		Enterococcus faecalis	1	3
		Enterococcus spp	0	2
		Gemella morbillorum	0	1
		Granulicatella spp	0	1
		Micrococcus spp	0	1
		Staphylococcus spp	0	2
TOTAL			29	41

APÊNDICE 3

Quantificação de Metaloproteinases da matriz (pg/mL) em dentes com insucesso do tratamento endodôntico

SUBSTÂNCIA	MMP-2			MMP-3			MMP-8			MMP-9			MMP-13		
	Inicial	PQM	MIC	Inicial	PQM	MIC	Inicial	PQM	MIC	Inicial	PQM	MIC	Inicial	PQM	MIC
CLOREXIDINA 2% GEL	772	716	760	779	198	221	332	301	267	140	93	86	52	75	74
	781	922	931	496	218	611	226	1830	302	135	74	38	93	92	79
	741	709	696	817	226	201	325	307	348	108	87	113	62	81	85
	734	878	672	310	248	212	167	384	284	154	158	40	94	106	84
	777	764	712	886	245	849	237	273	238	64	16	272	66	75	74
	905	800	731	227	224	212	141	3618	268	134	70	70	76	88	82
	751	677	681	411	231	480	268	278	280	64	85	129	61	79	76
	852	919	855	302	345	203	157	336	296	114	109	49	81	108	105
	1039	800	796	195	959	632	641	275	149	155	59	149	65	91	93
	1078	947	806	694	349	236	267	403	255	173	92	155	91	105	107
NaOCl 6%	764	707	798	277	263	255	134	191	111	89	303	92	54	38	56
	733	898	807	207	342	526	177	2643	173	140	133	457	66	86	57
	786	725	757	217	847	250	241	1354	142	151	53	102	56	53	56
	796	877	817	340	238	271	296	244	199	151	111	70	63	82	51
	748	662	708	529	224	357	173	188	320	133	93	90	69	78	79
	744	759	762	751	220	147	112	353	191	150	192	100	88	115	93
	823	670	742	330	199	249	405	292	161	147	69	65	65	71	70
	704	747	741	699	292	278	131	1220	170	115	97	110	79	93	71
	812	620	722	185	184	223	140	8242	188	160	119	182	60	68	118
	734	721	673	426	802	182	348	136	123	111	232	89	74	81	70
Média (G1)	843	813	764	512	324	386	276	801	269	124	84	110	74	90	86
Desvio Padrão (G1):	125	98	83	261	229	239	144	1099	52	37	36	72	15	13	12
Média (G2)	764	739	753	396	361	274	216	1486	178	135	140	136	67	77	72
Desvio Padrão (G2):	39	89	46	203	249	105	102	2506	58	23	79	117	11	21	21
Média (total)	804	776	758	454	343	330	246	1143	223	129	112	123	71	83	79
Desvio Padrão (total):	99	99	66	235	233	188	126	1916	71	30	66	96	13	18	18

Inicial, coleta após desobturação; PQM, coleta após o preparo químico-mecânico; MIC, coleta após a medicação intracanal; MMP, metaloproteinases da matriz.

APÊNDICE 4

Quantificação de bactérias cultiváveis (UFC/mL), LTA (pg/mL), TNF-α (pg/mL), IL-1β (pg/mL) nas diferentes fases do retratamento endodôntico.

SUBSTÂNCIA	Bactérias			LTA			TNF-α			IL-1β		
	Inicial	PQM	MIC	Inicial	PQM	MIC	Inicial	PQM	MIC	Inicial	PQM	MIC
CLOREXIDINA 2% GEL	155	0	3	112	53.6	16.2	10.4	3	7.7	2	0.04	1.0
	191	0	0	130	45.7	7.1	3.2	0	1.6	1	0.11	0.8
	238	0	0	111	37.2	47.9	1.3	1	0.2	1	0.07	0.5
	32	0	0	21.6	9.9	22	11.2	2	8.4	1	0.03	0.7
	26	0	1	70.2	17	45.1	6.6	1	1.4	1	0.42	0.3
	82	0	0	56.3	118	43.5	12.1	0	2.3	1	0.08	1
	32	0	0	85.6	81.7	18	7.2	0	1.0	1	0.21	0.9
	169	0	0	310	66.9	16.4	9.7	0	2.4	1	0.11	0.5
	123	0	0	85.8	27.2	48.8	6	0	1.4	2	0.01	0.9
	243	0	0	89.5	19.3	8.6	10.4	2	2.8	1	0.2	0.6
NaOCl 6%	25	0	0	38.5	25.7	5.8	7.8	0	1.9	1	0.01	1.0
	3	0	0	84.7	45.9	14.3	3.3	0	0.1	1	0.03	0.4
	12	0	1	66.8	3.7	40.5	17	0	3.0	1	0.07	0.8
	14	4	1	53.2	1.9	29.3	12.8	1	2.5	1	0.16	0.9
	117	5	2	40.6	26.9	11.5	3.19	3	0.2	2	0.36	0.8
	82	0	1	194	62	3.7	17.7	2	2.4	1	0.03	0.4
	152	2	0	63.9	48.5	19.4	6	1	4.6	1	0.09	0.8
	12	0	0	69.6	3.8	9	14.8	1	17.8	1	0.07	0.8
	145	0	1	87.4	9.8	22.9	4	0	3.7	1	0.21	0.8
	170	0	0	111	34.6	19.5	10.5	0	0.0	1	0.09	0.6
Média (G1)	129	0	0	107	48	27	8	1	3	1	0	1
Desvio Padrão (G1):	83	0	1	78	34	17	4	1	3	1	0	0
Média (G2)	73	1	1	81	26	18	10	1	4	1	0	1
Desvio Padrão (G2):	67	2	1	45	21	11	6	1	5	0	0	0
Média (total)	101	1	1	94	37	22	9	1	3	1	0	1
Desvio Padrão (total):	79	1	1	63	30	15	5	1	4	0	0	0

LTA, ácido lipoteicóico; TNF-α, fator de necrose tumoral alpha; IL-1β, interleucina 1-beta; Inicial, coleta após desobturação; PQM, coleta após o preparo químico-mecânico; MIC, coleta após a medicação intracanal.

ANEXO 1

COMITÊ DE ÉTICA EM PESQUISA
FACULDADE DE ODONTOLOGIA DE PIRACICABA
UNIVERSIDADE ESTADUAL DE CAMPINAS

CERTIFICADO

O Comitê de Ética em Pesquisa da FOP-UNICAMP certifica que o projeto de pesquisa **"ESTUDO CLÍNICO DA COMUNIDADE MICROBIANA E DOS ASPECTOS IMUNOBIOLÓGICOS ENVOLVIDOS NAS ALTERAÇÕES PULPARES E PERIRRADICULARES"**, protocolo nº 018/2014, dos pesquisadores Brenda Paula Figueiredo de Almeida Gomes, Ariane Cássia Salustiano Marinho, Daniel Rodrigo Herrera Morante, Marlos Barbosa Ribeiro e Thais Mageste Duque, satisfaz as exigências do Conselho Nacional de Saúde - Ministério da Saúde para as pesquisas em seres humanos e foi aprovado por este comitê em 07/05/2014, com alterações em 10/09/2014.

The Ethics Committee in Research of the Piracicaba Dental School - University of Campinas, certify that the project **"CLINICAL STUDY OF THE MICROBIAL COMMUNITY AND IMMUNOBIOLOGIC ASPECTS INVOLVED IN THE PULP AND PERIAPICAL DISEASES"**, register number 018/2014, of Brenda Paula Figueiredo de Almeida Gomes, Ariane Cássia Salustiano Marinho, Daniel Rodrigo Herrera Morante, Marlos Barbosa Ribeiro and Thais Mageste Duque, comply with the recommendations of the National Health Council - Ministry of Health of Brazil for research in human subjects and therefore was approved by this committee on May 07, 2014; with alterations on Sep 10, 2014.

Prof. Dr. Felippe Bevilacqua Prado
Secretário
CEP/FOP/UNICAMP

Profa. Dra. Lívia Maria Andaló Tenuta
Coordenadora
CEP/FOP/UNICAMP

Nota: O título do protocolo aparece como fornecido pelos pesquisadores, sem qualquer edição.
Notice: The title of the project appears as provided by the authors, without editing.

ANEXO 2

International Endodontic Journal
The Official Journal of the British Endodontic Society and the European Society of Endodontology

Effectiveness of root canal procedures on the reduction proinflammatory cytokines and matrix metalloproteinases in cases of post-treatment apical periodontitis

Journal:	*International Endodontic Journal*
Manuscript ID	Draft
Manuscript Type:	Original Scientific Article
Keywords:	Bacteria, Chlorhexidine, Sodium hypochlorite, Enterococcus faecalis, Cytokines, Matrix metalloproteinases